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(54) Title: SALMONELLA VACCINES

(57) Abstract

A bacterial cell the virulence of which is attenuated by a first mutation in a PhoP regulon and a second mutation in an aromatic amino acid synthetic gene and bacterial cells the virulence of which is attenuated by a mutation in one or more PhoP-activated genes or one or more PhoP-repressed genes.

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SALMONELLA VACCINES

Background of the Invention

The invention relates to vaccines.

This invention was made with Government support under Grant No. AI30479 and Grant No. 00917 awarded by the National Institutes of Health. The Government has certain rights in the invention.

Enteric fevers and diarrheal diseases, e.g., typhoid fever and cholera, are major causes of morbidity and mortality throughout the developing world, Hook et al., 1980, In Harrison's Principles of Internal Medicine, 9th Ed., 641-848, McGraw Hill, New York. Traditional approaches to the development of vaccines for bacterial diseases include the parenteral injection of purified components or killed organisms. These parenterally administered vaccines require technologically advanced preparation, are relatively expensive, and are often, because of dislike for needle-based injections, resisted by patients. Live oral vaccine strains have several advantages over parenteral vaccines: low cost, ease of administration, and simple preparation.

The development of live vaccines has often been limited by a lack of understanding of the pathogenesis of the disease of interest on a molecular level. Candidate live vaccine strains require nonrevertable genetic alterations that affect the virulence of the organism, but not its induction of an immune response. Work defining the mechanisms of toxigenesis of *Vibrio cholerae* has made it possible to create live vaccine strains based on deletion of the toxin genes, Mekalanos et al., 1983, Nature 306:551, Levine et al., 1988, Infect. Immun. 56:161.

Recent studies have begun to define the molecular basis of *Salmonella typhimurium* macrophage survival and

virulence, Miller et al., 1989, Proc. Natl. Acad. Sci. USA 86:5054, hereby incorporated by reference. *Salmonella typhimurium* strains with mutations in the positive regulatory regulon *phoP* are markedly attenuated in virulence for BALB/c mice. The *phoP* regulon is composed of two genes present in an operon, termed *phoP* and *phoQ*. The *phoP* and *phoQ* gene products are highly similar to other members of bacterial two-component transcriptional regulators that respond to environmental stimuli and control the expression of a large number of other genes. A mutation at one of these *phoP* regulatory region regulated genes, *pagC*, confers a virulence defect. Strains with *pagC*, *phoP*, or *phoQ* mutations afford partial protection to subsequent challenge by wild-type *S. typhimurium*.

Salmonella species cause a spectrum of clinical disease that includes enteric fevers and acute gastroenteritis, Hook et al., 1980, *supra*. Infections with *Salmonella* species are more common in immunosuppressed persons, Celum et al., 1987, J. Infect. Dis. 156:998. *S. typhi*, the bacterium that causes typhoid fever, can only infect man, Hook et al., 1980, *supra*. The narrow host specificity of *S. typhi* has resulted in the extensive use of *S. enteriditis typhimurium* infection of mice as a laboratory model of typhoid fever, Carter et al., 1984 J. Exp. Med. 139:1189. *S. typhimurium* infects a wider range of hosts, causing acute gastroenteritis in man and a disease similar to typhoid fever in the mouse and cow.

Salmonella infections are acquired by oral ingestion. The organisms, after traversing the stomach, replicate in the small bowel, Hornik et al., 1970, N. Eng. J. Med. 283:686. *Salmonella* are capable of invasion of the intestinal mucosal cells, and *S. typhi* can pass through this mucosal barrier and spread via the Peyer's

patches to the lamina propria and regional lymph nodes. Colonization of the reticuloendothelial cells of the host then occurs after bacteremia. The ability of *S. typhi* to survive and replicate within the cells of the human reticuloendothelial system is essential to its pathogenesis, Hook et al., 1980, *supra*, Hornick et al., 1970, *supra*, and Carter et al., 1984, *supra*.

Immunity to *Salmonella typhi* involves humoral and cell-mediated immunity, Murphy et al., 1987, J. Infect. Dis. 156:1005, and is obtainable by vaccination, Edelman et al., 1986, Rev. Inf. Dis. 8:324. Recently, human field trials demonstrated significant protective efficacy against *S. typhi* infection after intramuscular vaccination with partially purified Vi antigen, Lanata et al., 1983, Lancet 2:441. Antibody-dependent enhancement of *S. typhi* killing by T cells has been demonstrated in individuals who received a live *S. typhi* vaccine, indicating that these antibodies may be necessary for the host to generate a cell-mediated immune response, Levine et al., 1987, J. Clin. Invest. 79:888. The cell-mediated immune response is important in typhoid immunity since killed vaccines that do not induce this immune response are not protective in man, Collins et al., 1972, Infect. Immun. 41:742.

Summary of the Invention

The invention provides a *Salmonella* vaccine which does not cause transient bacteremia. In general, the invention features a bacterial cell, preferably a *Salmonella* cell, e.g., a *S. typhi*, *S. enteritidis typhimurium*, or *S. cholerae-suis* cell, the virulence of which is attenuated by a first mutation in a PhoP regulon and a second mutation in an aromatic amino acid synthetic gene. As used herein, PhoP regulon is defined as a DNA which comprises a unit of *Salmonella* virulence gene expression characterized by two regulatory genes, *phoP*

and *phoQ*, and structural genes, the expression of which is regulated by *phoP* and *phoQ*, e.g., phoP regulatory region repressed genes (prg) or phoP regulatory region activated genes (pag). Such a bacterial cell can be used as a vaccine to immunize a mammal against salmonellosis.

The *Salmonella* cell may be of any serotype, e.g., *S. typhimurium*, *S. paratyphi A*, *S. paratyphi B*, *S. paratyphi C*, *S. pylorum*, *S. dublin*, *S. heidelberg*, *S. newport*, *S. minnesota*, *S. infantis*, *S. virchow*, or *S. panama*.

The first mutation may be a non-revertable null mutation in the PhoP/PhoQ locus. Preferably, the mutation is a deletion of at least 100 nucleotides; more preferably, the mutation is a deletion of at least 500 nucleotides; even more preferably, the mutation is a deletion of at least 750 nucleotides; and, most preferably, the mutation is a deletion of nucleotides 376 to 1322 of the *PhoP/PhoQ* regulatory locus.

The second mutation may be a non-revertable null mutation in an *aroA* locus or a non-revertable null mutation in an *aroC/aroD* locus, or another locus involved in the biosynthesis of aromatic amino acids.

To further attenuate the virulence of the bacterial cell of the invention, the cell may contain yet another mutation, e.g., a deletion, in a non-aromatic amino acid synthetic gene, e.g., a mutation which renders the cell auxotrophic for a non-aromatic amino acid, e.g., histidine. In preferred embodiments, the bacterial cell of the invention is a *S. typhi* cell with the genotype $AroA^-$, His^-, $PhoP/PhoQ^-$, e.g., TyLH445.

The invention may also include a *Salmonella* cell, the virulence of which is attenuated by the constitutive expression of a gene under the control of a two-component regulatory system. In preferred embodiments the constitutive expression is the result of a mutation at a

component of the two-component regulatory system. In preferred embodiments the bacterial cell includes a second mutation which attenuates virulence.

In yet other preferred embodiments of the vaccine the two-component regulatory system is the phoP regulatory region, and the gene under the control of the two-component system is a phoP regulatory region regulated gene, e.g., a *prg* gene, e.g., *prgA*, *prgB*, *prgC*, *prgE*, or *prgH*, or *pag* gene, e.g., *pag*C. In preferred embodiments constitutive expression is the result of a change or mutation, e.g., a deletion, (preferably a non-revertible mutation) at the promoter of the regulated gene or of the phoP regulatory region, e.g., a mutation in the *pho*Q or the *pho*P gene, e.g., the PhoP^c mutation.

In another aspect, the invention features a vaccine including a bacterial cell which is attenuated by decrease of expression of a virulence gene under control of a *phoP* regulatory region, e.g., a *prg* gene, e.g., *prgA*, *prgB*, *prgC*, *prgE*, or *prgH*.

In preferred embodiments of the vaccine the *Salmonella* cell includes a first mutation, e.g., a deletion, which attenuates virulence, e.g., a mutation in a *pho*P regulatory region gene, e.g., a mutation in the *pho*P or *pho*Q gene, e.g., PhoP^c, or a mutation in a phoP regulatory region regulated gene, and a second mutation which attenuates virulence, e.g., a mutation in an *aro*matic amino acid synthetic gene, e.g., an *aro* gene, a mutation in a phoP regulatory region regulated gene, e.g., a mutation in a *prg* gene, e.g., *prgA*, *prgB*, *prgC*, *prgE*, or *prgH*, or *pag* locus, e.g., a *pag*C mutation.

In yet other preferred embodiments the bacterial cell includes a first mutation in a phoP regulatory region gene and a second mutation in an *aro*matic amino acid synthetic gene, e.g, an *aro* gene.

In another aspect, the invention features a

vaccine, preferably a live vaccine, including a bacterial cell, the virulence of which is attenuated by a mutation, e.g., a deletion, in a gene under the control of a two-component regulatory system. In preferred embodiments the bacterial cell includes a virulence attenuating mutation in a second gene, e.g., in an *aro*matic amino acid synthetic gene, e.g., an *aro* gene.

In yet other preferred embodiments of the vaccine the bacterial cell is a *Salmonella* cell, the two-component regulatory system is the phoP regulatory region, and the gene under its control is a *prg* gene, e.g. *prgA*, *prgB*, *prgC*, *prgE*, or *prgH*, or a *pag* gene, e.g., the *pagC* gene.

In another aspect the invention features a vaccine, preferably a live vaccine, including a *Salmonella* cell e.g., a *S. typhi*, *S. enteritidis typhimurium*, or *S. cholerae-suis* cell, including a first virulence attenuating mutation in an *aro*matic amino acid biosynthetic gene, e.g., an *aro* gene, and a second virulence attenuating mutation in a phoP regulatory region gene, e.g., a *pho*P$^-$ mutation.

In another aspect the invention features a bacterial cell, or a substantially purified preparation thereof, preferably a *Salmonella* cell, e.g., a *S. typhi*, *S. enteritidis typhimurium*, or *S. cholerae-suis* cell, which constitutively expresses a gene under the control of a two-component regulatory system and which includes a virulence attenuating mutation, e.g., a deletion, which does not result in constitutive expression of a gene under the control of the two-component regulatory system. In preferred embodiments the bacterial cell includes a mutation in a component of the two-component regulatory system.

In preferred embodiments the bacterial cell is a *Salmonella* cell which expresses a *pho*P regulatory region

regulated gene constitutively (the constitutive expression preferably caused by a mutation, preferably a non-revertible mutation, e.g., a deletion in the phoP regulatory region, e.g., a mutation in the *phoQ* or *phoP* gene, e.g., $phoP^c$), and which includes a virulence attenuating mutation, preferably a non-revertible mutation, e.g., a deletion, preferably in an aromatic amino acid synthetic gene, e.g., an *aro* gene, or in a phoP regulatory region regulated gene, e.g., a *prg* gene, e.g., *prgA*, *prgB*, *prgC*, *prgE*, or *prgH* or *pag* gene, e.g., *pagC* which does not result in the constitutive expression of a gene under the control of the phoP regulatory region.

In another aspect, the invention features a bacterial cell, or a substantially purified preparation thereof, e.g., a *Salmonella* cell, e.g., a *S. typhi* cell, an *S. enteritidis typhimurium* or a *S. cholerae-suis* cell, including a virulence attenuating mutation in a gene regulated by a two-component regulatory system. In preferred embodiments the virulence attenuating mutation is in a phoP regulatory region regulated gene, e.g., a *prg* gene, e.g., *prgA*, *prgB*, *prgC*, *prgE*, or *prgH* or *pag* gene, e.g., *pagC*.

In preferred embodiments the bacterial cell includes a second mutation, e.g., in an aromatic amino acid synthetic gene, e.g., an *aro* gene, in a phoP regulatory region gene, e.g., the phoP or phoQ genes, or in a phoP regulating region regulated gene, e.g., a *prg* gene, e.g., *prgA*, *prgB*, *prgC*, *prgE*, or *prgH* or a *pag* gene, e.g., *pagC*, which attenuates virulence but which does not result in constitutive expression of a phoP regulatory region regulated gene.

The invention also features a live *Salmonella* cell, or a substantially purified preparation thereof, e.g., a *S. typhi*, *S. enteriditis typhimurium*, or

S. cholerae-suis cell, in which there is inserted into a virulence gene, e.g., a gene in the phoP regulating region, or a phoP regulating region regulated gene, e.g., a *prg* gene, e.g., *prgA*, *prgB*, *prgC*, *prgE*, or *prgH* or a *pag* locus, e.g., *pag*C, a gene encoding a heterologous protein, or a regulatory element thereof.

In preferred embodiments the live *Salmonella* cell carries a second mutation, e.g., an *aro* mutation, e.g., an *aro*A mutation, e.g., *aro*A$^-$ or *aro*ADEL407, that attenuates virulence.

In preferred embodiments the DNA encoding a heterologous protein is under the control of an environmentally regulated promoter. In other preferred embodiments the live *Salmonella* cell further includes a DNA sequence encoding T7 polymerase under the control of an environmentally regulated promoter and a T7 transcriptionally sensitive promoter, the T7 transcriptionally sensitive promoter controlling the expression of the heterologous antigen.

The invention also features a vector capable of integrating into the chromosome of *Salmonella* including: a first DNA sequence encoding a heterologous protein; a second (optional) DNA sequence encoding a marker e.g., a selective marker, e.g., a gene that confers resistance for a heavy metal resistance or a gene that complements an auxotrophic mutation carried by the strain to be transformed; and a third DNA sequence, e.g., a *pho*P regulon encoded gene, e.g., a *prg* gene, e.g., *prgA*, *prgB*, *prgC*, *prgE*, or *prgH* or a *pag* locus, e.g., *pag*C, encoding a *pho*P regulatory region regulated gene product necessary for virulence, the third DNA sequence being mutationally inactivated.

In other preferred embodiments: the first DNA sequence is disposed on the vector so as to mutationally inactivate the third DNA sequence; the vector cannot

replicate in a wild-type *Salmonella* strain; the heterologous protein is under the control of an environmentally regulated promoter; and the vector further includes a DNA sequence encoding T7 polymerase under the control of an environmentally regulated promoter and a T7 transcriptionally sensitive promoter, the T7 transcriptionally sensitive promoter controlling the expression of the heterologous antigen.

In another aspect the invention includes a method of vaccinating an animal, e.g., a mammal, e.g., a human, against a disease caused by a bacterium, e.g., *Salmonella*, including administering a vaccine of the invention.

The invention also includes a vector including DNA which encodes the *pagC* gene product; a cell transformed with the vector; a method of producing the *pagC* gene product including culturing the transformed cell and purifying the *pagC* gene product from the cell or culture medium; and a purified preparation of the *pagC* gene product.

In another aspect the invention includes a method of detecting the presence of *Salmonella* in a sample including contacting the sample with *pagC* encoding DNA and detecting the hybridization of the *pagC* encoding DNA to nucleic acid in the sample.

The invention also includes a vector including DNA which encodes the *prgH* gene product; a cell transformed with the vector; a method of producing the *prgH* gene product including culturing the transformed cell and purifying the *prgH* gene product from the cell or culture medium; and a purified preparation of the *prgH* gene product.

In another aspect the invention includes a method of detecting the presence of *Salmonella* in a sample including contacting the sample with *prgH* encoding DNA

and detecting the hybridization of the *prgH* encoding DNA to nucleic acid in the sample.

In another aspect the invention features a method of attenuating the virulence of a bacterium, the bacterium including a two-component regulatory system, including causing a gene under the control of the two-component system to be expressed constitutively. In preferred embodiments the bacterium is *Salmonella*, e.g., *S. typhi*, *S. enteritidis typhimurium*, or *S. choleraesuis*, and the two-component system is the phoP regulatory region.

In yet another aspect, the invention features a substantially pure DNA which includes the sequence given in SEQ ID NO:5 or a fragment thereof.

The invention also includes a substantially pure DNA which includes a sequence encoding *pagD*, e.g., nucleotides 91 to 354 of SEQ ID NO:5 (*pagD* open reading frame (ORF)) and degenerate variants thereof that encode a product with essentially the amino acid sequence given in SEQ ID NO:6, as well as the *pagD* ORF and its 5′ non-coding region, nucleotides 4 to 814 of SEQ ID NO:15) which contains the *pagD* promoter. DNA in the region between the *pagC* ORF and the *pagD* ORF (nucleotides 4 to 814 of SEQ ID NO:15), DNA which includes the *pagC* promoter (nucleotides 562 to 814 of SEQ ID NO:15), and DNA which includes the *pagD* promoter alone (nucleotides 4 to 776 of SEQ ID NO:15) are also within the claimed invention.

The invention also includes a substantially pure DNA which includes a sequence encoding *envE*, e.g., nucleotides 1114 to 1650 of SEQ ID NO:5 (*envE* ORF) and degenerate variants thereof that encode a product with essentially the amino acid sequence given in SEQ ID NO:7.

Another aspect of the invention features a substantially pure DNA which includes a sequence encoding

msgA, e.g., nucleotides 1825 to 2064 of SEQ ID NO:5 (*msgA* ORF) and degenerate variants thereof which encode a product with essentially the amino acid sequence given in SEQ ID NO:8, as well as the *msgA* ORF with its 5′ non-coding region, nucleotides 1510 to 1824 of SEQ ID NO:5 containing the *msgA* promoter. Also within the invention is a substantially pure DNA comprising the *msgA* promoter alone (nucleotides 1510 to 1760 of SEQ ID NO:5).

In yet another aspect, the invention features a substantially pure DNA which includes a sequence encoding *envF*, e.g., nucleotides 2554 to 3294 of SEQ ID NO:5 (*envF* ORF) and degenerate variants thereof which encode a product with essentially the amino acid sequence given in SEQ ID NO:9, as well as the *envF* ORF with its 5′ non-coding region, nucleotides 2304 to 2553 of SEQ ID NO:5 which contains the *envF* promoter.

Also within the invention is a substantially pure DNA which includes the sequence given in SEQ ID NO:10 or a fragment thereof.

The invention also includes a substantially pure DNA which includes a sequence encoding *prgH*, e.g., nucleotides 688 to 1866 of SEQ ID NO:10 (*prgH* ORF) and degenerate variants thereof which encode a product with essentially the amino acid sequence given in SEQ ID NO:11, as well as the *prgH* ORF with its promoter region (nucleotides 1 to 689 of SEQ ID NO:10).

The invention also includes a substantially pure DNA which includes a sequence encoding *prgI*, e.g., nucleotides 1891 to 2133 of SEQ ID NO:10 (*prgI* ORF) and degenerate variants thereof which encode a product with essentially the amino acid sequence given in SEQ ID NO:12, as well as the *prgI* ORF with its promoter region (nucleotides 1 to 689 of SEQ ID NO:10.

In another aspect, the invention features a substantially pure DNA which includes a sequence encoding

prgJ e.g., nucleotides 2152 to 2457 of SEQ ID NO:10 (*prgJ* ORF) and degenerate variants thereof which encode a product with essentially the amino acid sequence given in SEQ ID NO:13, as well as the *prgJ* ORF and its promoter region (nucleotides 1 to 689 of SEQ ID NO:10.

In yet another aspect, the invention features a substantially pure DNA which includes a sequence encoding *prgK*, e.g., nucleotides 2456 to 3212 of SEQ ID NO:10 (*prgK* ORF) and degenerate variants thereof which encode a product with essentially the amino acid sequence given in SEQ ID NO:14, as well as the *prgK* ORF with its promoter region (nucleotides 1 to 689 of SEQ ID NO:10.

The invention also encompasses a bacterial cell the virulence of which is attenuated by a mutation, e.g., a deletion, in one or more genes selected from the group consisting of *pagD*, *pagE*, *pagF*, *pagG*, *pagH*, *pagI*, *pagJ*, *pagK*, *pagL*, *pagM*, *pagN*, *pagP*, *envE*, and *envF*. Also included is a bacterial cell which is attenuated by a mutation, e.g, a deletion, in one or more genes selected from the group consisting of *pagC*, *pagD*, *pagJ*, *pagK*, *pagM*, and *msgA*. A bacterial cell, the virulence of which is attenuated by a mutation, e.g., a deletion, in one or more genes selected from the group consisting of *prgH*, *prgI*, *prgJ*, and *prgK* is also within the claimed invention.

Two-component regulatory system, as used herein, refers to a bacterial regulatory system that controls the expression of multiple proteins in response to environmental signals. The two-components referred to in the term are a sensor, which may, e.g., sense an environmental parameter and in response thereto promote the activation, e.g. by promoting the phosphorylation, of the second component, the activator. The activator affects the expression of genes under the control of the two-component system. A two-component system can

include, e.g., a histidine protein kinase and a phosphorylated response regulator, as is seen in both gram positive and gram negative bacteria. In *E. coli*, e.g., 10 kinases and 11 response regulators have been identified. They control chemotaxis, nitrogen regulation, phosphate regulation, osmoregulation, sporulation, and many other cellular functions, Stock et al., 1989 Microbiol. Rev. 53:450-490, hereby incorporated by reference. A two-component system also controls the virulence of *Agrobacterium tumefasciens* plant tumor formation, Leroux et al. EMBO J 6:849-856, hereby incorporated by reference). Similar virulence regulators are involved in the virulence of *Bordetella pertussis* Arico et al., 1989, Proc. Natl. Acad. Sci. USA 86:6671-6675, hereby incorporated by reference, and *Shigella flexneri*, Bernardini et al., 1990, J. Bact. 172:6274-6281, hereby incorporated by reference.

Environmentally regulated, as used herein refers to a pattern of expression wherein the expression of a gene in a cell depends on the levels of some characteristic or component of the environment in which the cell resides. Examples include promoters in biosynthetic pathways which are turned on or off by the level of a specific component or components, e.g., iron, temperature responsive promoters, or promoters which are expressed more actively in specific cellular compartments, e.g., in macrophages or vacuoles.

A vaccine, as used herein, is a preparation including materials that evoke a desired biological response, e.g., an immune response, in combination with a suitable carrier. The vaccine may include live organism, in which case it is usually administered orally, or killed organisms or components thereof, in which case it is usually administered parenterally. The cells used for the vaccine of the invention are preferably alive and

thus capable of colonizing the intestines of the inoculated animal.

A mutation, as used herein, is any change (in comparison with the appropriate parental strain) in the DNA sequence of an organism. These changes can arise e.g., spontaneously, by chemical, energy e.g., X-ray, or other forms of mutagenesis, by genetic engineering, or as a result of mating or other forms of exchange of genetic information. Mutations include e.g., base changes, deletions, insertions, inversions, translocations or duplications.

A mutation attenuates virulence if, as a result of the mutation, the level of virulence of the mutant cell is decreased in comparison with the level in a cell of the parental strain, as measured by (a) a significant (e.g., at least 50%) decrease in virulence in the mutant strain compared to the parental strain, or (b) a significant (e.g., at least 50%) decrease in the amount of the polypeptide identified as the virulence factor in the mutant strain compared to the parental strain.

A non-revertible mutation, as used herein, is a mutation which cannot revert by a single base pair change, e.g., deletion or insertion mutations and mutations that include more than one lesion, e.g., a mutation composed of two separate point mutations.

The phoP regulatory region, as used herein, is a two-component regulatory system that controls the expression of *pag* and *prg* genes. It includes the phoP locus and the phoQ locus.

phoP regulatory region regulated genes, as used herein, refer to genes such as *pag* and *prg* genes.

pag, as used herein, refers to a gene which is positively regulated by the phoP regulatory region.

prg, as used herein, refers to a gene which is negatively regulated by the phoP regulatory region.

An *aro*matic amino acid synthetic gene, as used herein, is a gene which encodes an enzyme which catalyzes a step in the synthesis of an *aro*matic amino acid. *aro*A, *aro*C, and *aro*D are examples of such genes in *Salmonella*. Mutations in these genes can attenuate virulence without the total loss of immunogenicity.

Abnormal expressions, as used herein, means expression which is higher or lower than that seen in wild type.

Heterologous protein, as used herein, is a protein that in wild type, is not expressed or is expressed from a different chromosomal site, e.g., a heterologous protein is one encoded by a gene that has been inserted into a second gene.

Virulence gene, as used herein, is a gene the inactivation of which results in a *Salmonella* cell with less virulence than that of a similar *Salmonella* cell in which the gene is not inactivated. Examples include the phoP, *pag*C, *prgH* genes.

A marker, as used herein, is gene product the presence of which is easily determined, e.g., a gene product that confers resistance to a heavy metal or a gene product which allows or inhibits growth under a given set of conditions.

Purified preparation, as used herein, is a preparation, e.g., of a protein, which is purified from the proteins, lipids, and other material with which it is associated. The preparation is preferably at least 2-10 fold purified.

Constitutive expression, as used herein, refers to gene expression which is modulated or regulated to a lesser extent than the expression of the same gene in an appropriate control strain, e.g., a parental or in wild-type strain. For example, if a gene is normally repressed under a first set of conditions and derepressed

under a second set of conditions constitutive expression would be expression at the same level, e.g., the repressed level, the derepressed level, or an intermediate level, regardless of conditions. Partial constitutive expression is included within the definition of constitutive expression and occurs when the difference between two levels of expression is reduced in comparison in what is seen in an appropriate control strain, e.g., a wild-type or parental strain.

A substantially purified preparation of a bacterial cell is a preparation of cells wherein contaminating cells without the desired mutant genotype constitute less than 10%, preferably less than 1%, and more preferably less than 0.1% of the total number of cells in the preparation.

The invention allows for the attenuation of virulence of bacteria and of vaccines that include bacteria, especially vaccines that include live bacteria, by mutations in two-component regulatory systems and/or in genes regulated by these systems. The vaccines of the invention are highly attenuated for virulence but retain immunogenicity, thus they are both safe and effective. The vectors of the invention allow the rapid construction of strains containing DNA encoding heterologous proteins, e.g., antigens. The heterologous protein encoding DNA is chromosomally integrated, and thus stable, unlike plasmid systems which are dependent on antibiotic resistance or other selection pressure for stability. Live *Salmonella* cells of the invention in which the expression of heterologous protein is under the control of an environmentally responsive promoter do not express the heterologous protein at times when such expression would be undesirable e.g., during culture, vaccine preparation, or storage, contributing to the viability of the cells, but when administered to humans or animals, express large

amounts of the protein. This is desirable because high expression of many heterologous proteins in *Salmonella* can be associated with toxicity to the bacterium. The use of only a single integrated copy of the DNA encoding the heterologous protein also contributes to minimal expression of the heterologous protein at times when expression is not desired. In embodiments where a virulence gene, e.g., the *pag*C gene or the *prgH* gene, contains the site of integration for the DNA encoding the heterologous protein the virulence of the organism is attenuated.

A substantially pure DNA, as used herein, refers to a nucleic acid sequence, segment, or fragment, which has been purified from the sequences which flank it in a naturally occurring state, e.g., a DNA which has been removed from the sequences which are normally adjacent to the fragment, e.g., the sequences adjacent to the fragment in the genome in which it naturally occurs. The term also applies to DNA which has been substantially purified from other components which naturally accompany the DNA, e.g., DNA which has been purified from proteins which naturally accompany it in a cell.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments and from the claims.

Description of the Preferred Embodiments

The drawings will first be described.

Drawings

Fig. 1 is a graph of the survival of *Salmonella* strains within macrophages.

Fig. 2 is a map of the restriction endonuclease sites of the *pag*C locus.

Fig. 3 is a map of the DNA sequence of the *pag* C region (SEQ ID NO:1).

Fig. 4 is a map of the location of *prgH* within the

hil locus. The arrows indicate the direction of orientation of the neomycin promoter of Tn*5*B50 insertions within the *hil* locus and the direction of transcription of the *prgH1*::Tn*phoA* fusion protein. Restriction endonuclease sites are represented by B, *Bam*H1; H, *Hind*III; X, *Xho*I; S, *Sac*I; V, *Eco*RV.

Fig. 5 is a DNA sequence from the *prgH* gene (plasmid pIB01) (SEQ ID NO:3).

Fig. 6 is a bar graph showing a comparison of the sensitivity of wild type (ATCC 14028), PhoP-null mutant (CS015), and *pag*::Tn*phoA* mutant strains to NP-1 defensin. The y-axis represents the Defensin Killing Index (DKI) which is a measure of bacteria killed on exposure to NP-1. The DKI is defined as the logarithmic function of the ratio of control bacteria to surviving bacteria incubated with NP-1 [DKI=log (CFU without NP-1/CFU with NP-1)]. The individual bars represent the mean and standard error of five separate experiments. The x-axis indicates the allele mutated. The mean DKI for each of the *pag*::Tn*phoA* strains tested was determined not be different from that of wild type *Salmonella*. ($P<0.05$). In contrast, the *phoP* mutant was significantly different ($P<0.0001$).

Fig. 7 is a diagram showing a partial physical map of the restriction endonuclease sites of the *pagC* chromosomal region. The mouse 50% lethal doses (LD_{50}) for strains with transposon insertions in *pagD*, *envE*, *msgA*, and *pagC* are shown above each gene. Horizontal arrows demonstrate the direction of transcription. Vertical arrows denote Tn*phoA* insertions and the hollow triangle denotes a Mud*J* insertion. Below the chromosomal map is a representation of the DNA insert in plasmid pCAA9, which was mutagenized with Tn*phoA* and Mud*J*. Letter designations: A, *Acc*I; C, *Cla*I; E, *Eco*RI; H, *Hpa*I; P, *Pst*I; and V, *Eco*RV.

Fig. 8 is a DNA sequence of the region upstream of *pagC* and the translation of each ORF. The *Hpa*I and *Cla*I sites at the beginning and end of the region are indicated. Shine-Delgarno regions are underlined and stem loop structures (potential Rho-independent terminators) are indicated with a line below and above the sequence. Arrow heads denote the location of the representative transposon insertion within each gene. Horizontal arrows in the *pagD* and *msgA* promoter regions mark the transcriptional start sites, and asterisks mark the -10 and -35 sequences. The consensus lipid attachment site in EnvF is enclosed in brackets. The *pagD* ORF begins at nucleotide 91 and ends at nucleotide 354 of SEQ ID NO:5; the *envE* ORF begins at nucleotide 1114 and ends at nucleotide 1650 of SEQ ID NO:5; the *msgA* ORF begins at nucleotid 1825 and ends at nucleotide 2064 of SEQ ID NO:5; and the *envF* ORF begins at nucleotide 2554 and ends at nucleotide 3294 of SEQ ID NO:5.

Fig. 9 is a DNA sequence containing the *prgH*, *prgI*, *prgJ*, and *prgK* genes. The start codon (ATG) of each gene is underlined, and the stop codon is indicated with an asterisk. The *prgH* ORF begins at nucleotide 688 and ends at 1866 of SEQ ID NO:10; the *prgI* ORF begins at nucleotide 1891 and ends at nucleotide 2133 of SEQ ID NO:10; the *prgJ* ORF begins at nucleotide 2152 and ends at nucleotide 2457 of SEQ ID NO:10; and the *prgK* ORF begins at nucleotide 2454 and ends at nucleotide 3212 of SEQ ID NO:10.

Fig. 10 is a line graph showing the growth rates of the parent *Salmonella* strain (AroA-) and the vaccine strain (AroA-, PhoP-).

Fig. 11 is a bar graph showing defensin sensitivity of mouse vaccine strains (*S. typhimurium*).

Fig. 12 is a bar graph showing phoP activation as measured by LacZ activity using the PagB:LacZ recorder fusion construct.

Fig. 13 is a bar graph showing defensin sensitivity of *S. typhi* vaccine strain TyLH445 compared to the AroA$^-$ parent strain.

Fig. 14A is a graph showing the relative expression of constitutive expression (610 and 617) and phoP regulated (PagC and pagD) expression of AP fusion proteins.

Fig. 14B is a graph showing the immune response to lipopolysaccharide (LPS).

Fig. 14C is a graph showing the immune response to the model heterologous antigen, AP.

Fig. 15 is a DNA sequence containing the *pagC-pagD* intergenic region. *pagC* translational start site (ATG on the opposite DNA strand) is underlined (nucleotides 1-3 of SEQ ID NO:15). The *pagC* transcriptional start (nucleotide 562) is indicated with an arrow pointing left. The *pagD* translational start (ATG) is underlined (nucleotides 815-817 of SEQ ID NO:15). The *pagD* transcriptional start is indicated with an arrow pointing right (nucleotide 776).

Strain Deposit

Under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure, deposit of the following materials has been made with the American Type Culture Collection (ATCC) of Rockville, MD, USA.

Applicant's assignee, Massachusetts General Hospital, represents that the ATCC is a depository affording permanence of the deposit and ready accessibility thereto by the public if a patent is granted. All restrictions on the availability to the

public of the material so deposited will be irrevocably removed upon the granting of a patent. The material will be available during the pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 U.S.C. §122. The deposited material will be maintained with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposited plasmid, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of the patent, whichever period is longer. Applicants' assignee acknowledges its duty to replace the deposit should the depository be unable to furnish a sample when requested due to the condition of the deposit.

PhoP^{c} strain CS022 (described below) has been deposited with the American Type Culture Collection (Rockville, MD) and has received ATCC designation 55130.

The plasmid, pIB01, containing the *prgH* gene has been deposited on July 9, 1993 with the American Type Culture Collection (Rockville, MD) and has received ATCC designation ATCC 75496.

Constitutive Expression of the PhoP Regulon Attenuates Salmonella Virulence and Survival within Macrophages

The *phoP* constitutive allele (*PhoP*^{c}), *pho-24*, results in derepression of *pag* loci. Using diethyl sulfate mutagenesis of *S. typhimurium* LT-2, Ames and co-workers isolated strain TA2367 *pho-24* (all strains, materials, and methods referred to in this section are described below), which contained a *phoP* locus mutation that resulted in constitutive production of acid phosphatase in rich media, Kier et al., 1979, J. Bacteriol. 138:155, hereby incorporated by reference. This *phoP*-regulated acid phosphatase is encoded by the *phoN* gene, a *pag* locus, Kier et al., 1979, supra, Miller

et al., 1989, supra. To analyze whether the *pho-24* allele increased the expression of other *pag* loci the effect of the *pho-24* allele on the expression of other *pag* loci recently identified as transcriptional (e.g., *pag*A and *pag*B) and translational (e.g., *pag*C) fusion proteins that required *phoP* and *phoQ* for expression, Miller et al., 1989, supra, was determined. *pag* gene fusion strains, isogenic except for the *pho-24* allele, were constructed and assayed for fusion protein activity. $PhoP^c$ derivatives of the *pag*A::Mu dJ and *pag*B::Mu dJ strains produced 480 and 980 U, respectively, of β-galactosidase in rich medium, an increase of 9- to 10-fold over values for the fusion strains with a wild-type *phoP* locus, see Table 1.

The *pag*C::Tn*phoA* gene fusion produced 350 U of AP, an increase of three- to fourfold over that produced in strain CS119, which is isogenic except for the *pho-24* mutation, Miller et al., 1989, supra. These results compare with a ninefold increase in the acid phosphatase activity in strain CS022 on introduction of the *pho-24* allele. Therefore, these available assays for *pag* gene expression document that the *pho-24* mutation causes constitutive expression of *pag* loci other than *phoN*.

Table 1: Bacterial strains and properties

Strain	Genotype	Enzyme activity (U)[a]	Reference or source
10428	Wild type	180 (A)	ATCC; Miller et al., 1989, supra
TA2367	*pho-24*	1,925 (A)	Kier et al., 1974, supra
CS003	Δ*phoP* Δ*purB*	<10 (A)	Miller et al., 1989, supra
CS022	*pho-24*	1,750 (A)	This work
CS023	*pho-24 phoN2* *zxx*::6251Tn10d-Cam	25 (A)	This work
CS012	*pagA1*::MU dJ	45 (B)	Miller et al., 1989, supra
CS013	*pagB1*::MU dJ	120 (B)	Miller et al., 1989, supra
CS119	*pagC1*::Tn*phoA phoN2* *zxx*::6251Tn*10*d-Cam	85 (C)	Miller et al., 1989, supra
SC024	*pagA1*::Mu dJ *pho-24*	450 (B)	This work
SC025	*pagB1*::Mu dJ *pho-24*	980 (B)	This work
SC026	*pagC1*::Tn*phoApho-24phoN2* *zxx*::6251Tn*10*d-Cam	385 (B)	This work
CS015	*phoP102*::Tn*10*d-Cam	<10 (A)	Miller et al., 1989, supra
TT13208	*phoP105*::Tn*10*d	<10 (A)	--[b]

[a] A. Acid phosphatase; B, β-galactosidase; C, alkaline phosphatase (AP).

[b] Gift of Ning Zhu and John Roth.

Identifications of protein species that are repressed as well as activated in the PhoP^c mutant strain

Whole-cell proteins of strain CS022 were analyzed to estimate the number of protein species that could be potentially regulated by the PhoP regulon. Remarkably, analysis by one-dimensional polyacrylamide gel electrophoresis of the proteins produced by strains with the PhoP^c phenotype indicated that some protein species were decreased in expression when many presumptive *pag* gene products were fully induced by the *pho-24* mutation. The proteins decreased in the PhoP^c strain might represent products of genes that are repressed by the PhoP regulator. Genes encoding proteins decreased by the *pho-24* allele are designated *prg* loci, for *phoP*-repressed genes. Comparison of wild-type, PhoP$^-$, and PhoP^c mutant strain proteins shows that growth in LB medium at 37°C represents repressing conditions for *pag* gene products and derepressing conditions for *prg* gene products.

To estimate the total number of potentially PhoP-regulated gene products, the total cell proteins of wild-type and PhoP^c mutant strains grown in LB were analyzed by two-dimensional gel electrophoresis. At least 40 species underwent major fluctuation in expression in response to the *pho-24* mutation.

Virulence defects of the PhoP^c strain

Remarkably, strains with the single *pho-24* mutation were markedly attenuated for virulence in mice (Table 2). The number of PhoP^c organisms (2×10^5) that killed 50% of BALB/c mice challenged (LD_{50}) by the intraperitoneal (i.p.) route was near that (6×10^5) of PhoP$^-$ bacteria, Miller et al., 1989, supra. The PhoP^c strains had growth comparable to wild-type organisms in rich and minimal media. The PhoP^c mutants were also tested for alterations in lipopolysaccharide, which could explain the virulence defect observed. Strain CS022 had

Table 2
Virulence and protective efficacy of PhoP^c and PhoP$^-$ *Salmonella* strains

Immunizing dose	No. of initial survivors/ total	No. of survivors/total after wild-type challenge dose of: $5x10^7$	$5x10^5$	$5x10^4$	$5x10^3$
PhoP^c organisms					
15	13/13		5/5	4/5	
50	4/4				4/4
$1.5x10^2$	11/11		4/4	3/3	
$5x10^2$	16/16				/4
$1.5x10^3$	5/5		3/3	2/2	
$5x10^3$	4/4				4/4
$1.5x10^4$	5/5		3/3	2/2	
$5x10^4$	19/23				4/4
$1.5x10^5$	5/5		3/3	2/2	
$5x10^5$	1/4				1/1
$5x10^6$	0/6				
$3x10^9$(*)	5/5	5/5			
$3x10^{10}$(*)	5/5	5/5			
$1.5x10^{11}$(*)		5/5	5/5		
PhoP$^-$organisms					
$6x10^3$	36/36		0/12	0/12	0/12
$6x10^4$	36/36		0/12	0/12	3/12
$6x10^5$	19/36		0/6	0/6	4/7
$5x10^{10}$(*)	7/7	3/7			

(*) Organisms were administered by the oral route. In all other experiments, organisms were administered by i.p. challenge.

normal sensitivity to phage P22, normal group B reactivity to antibody to O antigen, and a lipopolysaccharide profile identical to that of the parent strain, as determined by polyacrylamide gel electrophoresis and staining.

Since the TA2367 *pho-24* strain was constructed by chemical mutagenesis and could have another linked mutation responsible for its virulence defect revertants of the PhoP^c were isolated to determine whether the *pho-24* allele was responsible for the attenuation of virulence observed. Phenotype PhoP^c revertants, identified by the normal levels of acid phosphatase in rich medium, were isolated among the bacteria recovered from the livers of mice infected with strain CS022. Six separate phenotypic revertants, designated CS122 to CS128, were found to be fully virulent (LD_{50} of less than 20 organisms for BALB/c mice). The locus responsible for the reversion phenotype was mapped in all six revertants tested for virulence by bacteriophage P22 cotransduction and had linkage characteristics consistent with the *phoP* locus (greater than 90% linkage to *purB*). These data indicate that these reversion mutations are not extragenic suppressors but are intragenic suppressors or true revertants of the *pho-24* mutation. Thus, the virulence defect of PhoP^c mutants is probably the result of a single revertible mutation in the *phoP* locus and not the result of a second unrelated mutation acquired during mutagenesis.

Reversion frequency of the PhoP^c phenotype

The reversion frequency of the PhoP^c mutation *in vivo* in mice was investigated to assess whether reversion could reduce the LD_{50} of this strain. The presence of the revertants of strain CS022 was tested for by administering 10^6, 10^4, and 10^2 challenge organisms to each of eight animals by i.p. injection. On day 7, three

animals died that received 10^6 PhoP^c organisms. On that day, the livers and spleens of all animals were harvested and homogenized in saline. After appropriate dilution, 10% of the tissue was plated on LB plates containing the chromogenic phosphatase substrate XP. Revertants were identified by their lighter blue colonies compared with PhoP^c bacteria and were confirmed by quantitative acid phosphatase assays. An estimated 10^7, 10^5, and 10^3 organisms per organ were recovered from animals at each of the three respective challenge doses. Revertants were identified only at the highest dose and comprised 0.5 to 1%, or 10^5 organisms per organ, at the time of death. It is likely that revertants are able to compete more effectively for growth in these macrophage-containing organs, since strain CS022 is deficient in survival within macrophages (see below). However, revertants were not identified if fewer than 10^5 organisms were administered in the challenge dose, suggesting that the reversion frequency must be approximately 10^{-5}. The reversion rate of the PhoP^c phenotype for CS022 bacteria grown in LB is in fact $6x10^{-4}$ when scored by the same colony phenotypes. The percentage of revertants recovered from animals near death suggests that pressure is applied *in vivo* that selects for revertants of the PhoP^c phenotype and implies that the virulence defect observed could be much greater quantitatively for a strain with a nonrevertible PhoP^c mutation.

The PhoP^c strain is deficient in survival within macrophages

Because of the importance of survival within macrophages to *Salmonella* virulence Fields et al., 1986, Proc. Natl. Acad. Sci. USA 83:5189, hereby incorporated by reference, PhoP^c bacteria were tested for this property. Strain CS022 was defective in the ability to grow and persist in macrophages as compared with wild-type organisms (Fig. 1). In Fig. 1 the survival of

strain CS022 ($PhoP^c$) (triangles) in cultured macrophages is compared with that of wild-type *S. typhimurium* ATCC 10428 (cicles). The experiment shown is a representative one. The difference between the two strains at 4 and 24 hours is significant ($P < 0.05$). $PhoP^-$ bacteria seemed to have a macrophage survival defect qualitatively similar to that of $PhoP^c$ bacteria but survived consistently better by two- to threefold in side-by-side experiments. The increased recovery of organisms that reverted to $PhoP^c$ phenotype in mouse organs rich in macrophage content is consistent with the reduced macrophage survival of $PhoP^c$ mutants in vitro.

Use of the $PhoP^c$ strain as a live vaccine

It has been previously reported that $PhoP^-$ strains are useful as live vaccines in protecting against mouse typhoid, Miller et al., 1989, supra. The immunogenicity of $PhoP^c$ when used as live attenuated vaccines in mice was compared with the of $PhoP^-$. This was done by simultaneous determination of survival, after graded challenge doses with the wild-type strain ATCC 10428, in mice previously immunized with graded doses of the two live vaccine strains. CS015 *phoP::Tn10d-Cam* and CS022 *pho-24*, as well as a saline control. The results obtained (Table 2) suggest the following conclusions: (i) small i.p. doses of the $PhoP^c$ strain (e.g., 15 organisms) effectively protect mice from challenge doses as large as $5x10^5$ bacteria (a challenge dose that represents greater than 10^4 i.p. LD_{50}s), (ii) large doses of $PhoP^c$ organisms given orally completely protect mice from an oral challenge consisting of $5x10^7$ wild-type bacteria (over 200 oral wild-type LD_{50}s) and (iii) by comparison, a large dose of $PhoP^-$ organisms ($5x10^5$) does not provide similar protection. The reversion of the $PhoP^c$ mutation in vivo somewhat complicates the analysis of the use of these strains as vaccines, since revertants of the CS022 strain

(i.e., wild-type cells) could increase immunogenicity). However, we were unable to identify revertants by examining 10% of the available spleen and liver tissue from those mice that received 10^4 or fewer organisms.

Strains, Materials and Methods

The strains, materials, and methods used in the PhoP regulon work described above are as follows.

American Type Culture Collection (ATCC) strain 14028, a smooth virulent strain of *S. typhimurium*, was the parent strain for all virulence studies. Strain TT13208 was a gift from Nang Zhu and John Roth. Strain TA2367 was a generous gift of Gigi Stortz and Bruce Ames, Kier et al., 1979, supra. Bacteriophage P22HT *int* was used in transductional crosses to construct strains isogenic except for *phoP* locus mutations, Davis et al., 1980, Advanced Bacterial Genetics, p. 78, 87. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, hereby incorporated by reference. Luria broth was used as rich medium, and minimal medium was M9, Davis et al., 1980, supra. The chromogenic phosphatase substrate 5-bromo-4-chloro-3indolyl phosphate (XP) was used to qualitatively access acid and AP production in solid media.

Derivatives of *S. typhimurium* ATCC 10428 with the *pho-24* mutation were constructed by use of strain TA2367 as a donor of the *purB* gene in a P22 transductional cross with strain CS003 Δ*phoP* Δ*purB*, Miller et al., 1989, supra. Colonies were then selected for the ability to grow on minimal medium. A transductant designated CS022 (phenotype $PhoP^c$) that synthesized 1,750 U of acid phosphatase in rich medium (a ninefold increase over the wild-type level in rich medium) was used in further studies.

Derivatives of strains CS022 and CS023 *pho-24 phoN2 zxx*::6251Tn*10*d-Cam, and acid phosphatase-negative derivative of CS022, containing *pag* gene fusions were

constructed by bacteriophage P22 transductional crosses, using selection of Tn*phoA*- or Mu dJ-encoded kanamycin resistance. Strains were checked for the intact *pag* gene fusion by demonstration of appropriate loss of fusion protein activity on introduction of a *phoP105*::Tn*10*d or *phoP102*::Tn*10*d-Cam allele.

Assays of acid phosphatase, AP, and β-galactosidase were performed as previously described, Miller et al., 1989, supra and are reported in units as defined in Miller, 1972, Experiments in molecular genetics, p. 352-355, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, hereby incorporated by reference.

In the mouse virulence and vaccination studies bacteria grown overnight in Luria broth were washed and diluted in normal saline. The wild-type parent strain of CS022 (ATCC 10428) was used for all live vaccine challenge studies. This strain has a 50% lethal dose (LD_{50}) for naive adult BALB/c mice of less than 20 organisms when administered by intraperitoneal (i.p.) injection and $5x10^4$ when administered orally in $NaHCO_3$. Mice were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) and were 5 to 6 weeks of age at initial challenge. All i.p. inoculations were performed as previously described, Miller et al., 1989, supra. Oral challenge experiments were performed with bacteria grown in LB broth and concentrated by centrifugation. The bacteria were resuspended in 0.1 M $NaHCO_3$ to neutralize stomach acid, and administered as a 0.5-ml bolus to animals under ether anesthesia. Colony counts were performed to accurately access the number of organisms administered. All challenge experiments were performed 1 month after i.p. inoculation and 6 weeks after oral challenge. Challenge inocula were administered by the same route as vaccinations. The care of all animals was under institutional guidelines as set

by the animal are committees at the Massachusetts General Hospital and Harvard Medical School.

Protein electrophoresis was performed as follows. One-dimensional protein gel electrophoresis was performed by the method of Laemmli, 1970, Nature 227:680, hereby incorporated by reference, on whole-cell protein extracts of stationary-phase cells grown overnight in Luria broth. The gels were fixed and stained with Coomassie brilliant blue R250 in 10% acetic acid-10% methanol. Two-dimensional protein gel electrophoresis was performed by method of O'Farrell, 1975, J. Biol. Chem. 250:4007, hereby incorporated by reference, on the same whole-cell extracts. Isoelectric focusing using 1.5% pH 3.5 to 10 ampholines (LKB Instruments, Baltimore, Md.) was carried out for 9,600 V h (700 V for 13 h 45 min). The final tube gel pH gradient extended from pH 4.1 to pH 8.1 as measured by a surface pH electrode (BioRad Laboratories, Richmond, Calif.) and colored acetylated cytochrome pI markers (Calbiochem-Behring, La Jolla, Calif.) run in an adjacent tube. The slab gels were silver stained, Merril et al., 1984, Methods Enzymol. 104:441, hereby incorporated by reference.

In the macrophage survival assays experiments were performed as previously described, Miller et al., 1989, supra, by the method of Buchmeier et al., 1989, Infect. Immun. 57:1, hereby incorporated by reference, as modified from the method of Lissner et al, 1983, J. Immunol. 131:3006, hereby incorporated by reference. Stationary-phase cells were opsonized for 30 min in normal mouse serum before exposure to the cultured bone marrow-derived macrophages harvested from BALB/c mice. One hour after infection, gentamicin sulfate (8 μg/ml) was added to kill extracellular bacteria. All time points were done in triplicate and repeated on three separate occasions.

PhoP^c Mutant Strains Are More Effective as Live Vaccines

PhoP^c mutant *S. typhimurium* are very effective when used as a live vaccine against mouse typhoid fever and are superior to PhoP$^-$ bacteria. As few a 15 PhoP^c bacteria protect mice against 10^5 LD_{50} (lethal doses 50%) of wild type organisms by the intraperitoneal route (Table 3). This suggests that *pag* gene products are important antigens for protective immunity against mouse typhoid. Preliminary results have documented that antigens recognized by serum of chronic typhoid carriers recognizes some *phoP*-regulated gene products of *S. typhi*. If protective antigens are only expressed within the host, then dead vaccines only grown in rich media may not induce an immune response against these proteins.

The use of different *S. typhimurium* dead vaccine preparations containing different mutations in the *phoP* regulon was evaluated. As can be seen in Table 3 no dead cell preparations (even those containing mixtures of PhoP$^-$ and PhoP^c bacteria) are as effective vaccines as are live bacteria. This suggests that there are other properties of live vaccines that increase immunogenicity or that important non-PhoP-regulated antigens are not in these preparations. The only protection observed in any animals studied was at the lowest challenge dose for those immunized with PhoP^c bacteria. This further suggests that *phoP* activated genes are important protective antigens.

Table 3

Salmonella with *phoP* regulon mutations used as a dead vaccine

Vaccination Strain	phenotype	Challenge dose of wild type organisms 6×10^3	6×10^5
None		(3)	(5)
ATCC10428	wild type	(8)	(9)
CS015	PhoP$^-$	(10)	(13)
CS022	PhoP^c	2/7(*)	(14)
CS022/CS015	PhoP$^-$/PhoP^c	(8)	(13)

CS015 = *phoP102*::Tn*10d-Cam*

CS022 = *pho-24*

BALB/c mice were immunized twice, 7 days apart, with 5×10^8 formalin-killed bacteria. Three weeks after the second vaccination, mice were challenged with wild-type organisms at the two doses indicated. The numbers in parentheses indicate no survivors after challenge and mean number of days until death

(*) Ratio of survivors to number challenged.

phoP^c indicates the constitutive unregulated expression of *phoP*-activated genes and lack of expression of *phoP* repressed genes.

phoP$^-$ indicates a lack of expression of *phoP*-activated genes and expression of *phoP* repressed genes.

aroA PhoP Regulon Double Mutant Strains

Recent efforts by Stocker, Levine, and colleagues have focused on the use of strains with auxotrophic mutations in *aro*matic amino acid and purine pathways as live vaccines, Hoseith et al., 1981, Nature 291:238, hereby incorporated by reference, Stocker, 1988, Vaccine 6:141, hereby incorporated by reference, and Levine et al., 1987, J. Clin. Invest. 79:888, hereby incorporated by reference. Purine mutations were found to be too attenuating for immunogenicity, likely because purines are not available to the organism within the mammalian host, Sigwart et al., 1989, Infect. Immun. 57:1858, hereby incorporated by reference. Because auxotrophic mutations may be complemented by homologous recombination events with wild type copies donated from environmental organisms or by acquiring the needed metabolite within the host, it would seem prudent for live vaccines to contain a second attenuating mutation in a different virulence mechanism, (i.e., not just a second mutation in the same metabolic pathway). Additionally, in mice the *aro*A mutants have some residual virulence. Various strains with *aro*A mutations combined with *phoP* regulon mutations were investigated for virulence attenuation and immunogenicity. Table 4 demonstrates that a $PhoP^-$ or $PhoP^c$ mutation further attenuates *aro*A mutant *S. typhimurium* by at least 100-fold and that, at least at high levels of vaccinating organisms, immunogenicity is retained. Strains with both a $pagC^-$ and $phoP^c$ phenotype are also further attenuated than either mutation alone. Therefore, *phoP* regulon mutations may increase the safety of *aro*A live vaccine preparations.

Table 4A

Additional attenuation of *aroA* mutants by PhoP regulon mutations

Strain	Phenotype	Survivors of varying numbers of *Salmonella* mutant organisms (*) 10^6	10^7	10^8	10^9	10^{10}(**)
CS004	*aroA*-	6/6	1/6	0/6	0/6	6/6
SL3261	*aroA*del His$^-$	6/6	1/6	0/6	0/6	6/6
CS322	*aroA*- PhoP^c	6/6	6/6	6/6	1/6	6/6
CS323	Sl3261 PhoP^c	6/6	6/6	6/6	2/6	6/6
CS315	*aroA*- PhoP$^-$	6/6	6/6	6/6	2/6	6/6
CS316	SL3261 PhoP$^-$	6/6	6/6	6/6	1/6	6/6
CS026	*pagC*$^-$ PhoP^c	6/6	4/6	0/6	0/6	6/6

Table 4B

Protective efficacy of *Salmonella* with *aroA/phoP* regulon mutations

Strain	Phenotype	Inoculum	Survivors of challenge doses of wild type organisms (*) 5 x 10^5	5 x 10^7
CS004	*aroA*$^-$	10^6	4/4	5/5
SL3261	*aroA*del His$^-$	10^6	4/4	4/5
CS322	*aroA*$^-$ PhoP^c	10^6	5/5	
CS323	SL3261 PhoP^c	10^6	5/5	
CS322	*aroA*$^-$ PhoP^c	10^7	5/5	
CS323	SL3261 PhoP^c	10^7	5/5	
CS322	*aroA*$^-$ PhoP^c	10^8		5/5
CS323	SL3261 PhoP^c	10^8		5/5
CS315	*aroA*$^-$ PhoP$^-$		5/5	
CS316	SI3261 PhoP$^-$	10^8	5/5	

(*) Ratio of survivors to number of mice challenged.
(**) Indicates oral inoculation all other experiments were intraperitoneal inoculation.
CS004 = *aro*A554::rn*10*.
SL3261 = *aro*ADEL407 *hisG46*.
CS322 = *aro*A554::Tn10 pho-24.
CS323 = *aro*ADEL407 pho-24.
CS315 = *aro*A554::Tn*10 phoP102*::Tn*10d-Cam*.
CS316 = *aro*ADEL407 hisG46 phoP102::Tn*10d-Cam*.
CS026 = *pag*C1::Tn*phoA pho-24 phoN2 zxx*::6251TN*10d-Cam*.

Salmonella typhi phoP Regulon Mutations

The *phoP* regulon is at least partially conserved in *S. typhi* DNA hybridization studies as well as P22 bacteriophage transductional crosses have documented that the *phoP*, *phoQ*, and *pagC* genes appear highly conserved between *S. typhi* and *S. typhimurium* mutations in these genes in *S. typhi* have been made.

Salmonella Live Vaccines as Delivery Systems for Heterologous Antigens

The vector used in the vaccine delivery system is a derivative of pJM703.1 described in Miller et al., 1988, J. Bact. 170:2575, hereby incorporated by reference. This vector is an R6K derivative with a deletion in the *pir* gene. R6K derivatives require the protein product of the *pir* gene to replicate. *E. coli* that contain the *pir* gene present as a lambda bacteriophage prophage can support the replication of this vector. Cells that do not contain the *pir* gene will not support the replication of the vector as a plasmid. This vector also contains the *mob* region of RP4 which will allow mobilization into other gram negative bacteria by mating from *E. coli* strains such as SM10lambda *pir*, which can provide the mobilization function in trans.

The *pagC* region is shown in Figs. 2 and 3. Fig. 2 shows the restriction endonuclease sites of the *pagC* locus. The heavy bar indicates *pagC* coding sequence. The Tn*phoA* insertion is indicated by a inverted triangle. The direction of transcription is indicated by the arrow and is left to right. The numbers indicate the location of endonuclease sites, in number of base pairs, relative to the start codon of predicted *pagC* translation with positive numbers indicating location downstream of the start codon and negative numbers indicating location upstream of the start codon. A is *Acc*I, B is *Bgl*II, C is *Cla*I, D is *Dra*I, E is *Eco*RI, H is *Hpa*I, N is *Nru*I, P is *Pst*I, S is *Ssp*I, T is *Stu*I, U is *Pvu*II, V is *Eco*RV, and

II is *Bg1*II. Fig. 3 shows the DNA sequence (Sequence I.D. No. 1) and translation of *pagC*::Tn*phoA*. The heavy underlined sequence indicates a potential ribosomal binding site. The single and double light underlines indicate sequences in which primers were constructed complementary to these nucleotides for primer extension of RNA analysis. The asterisk indicates the approximate start of transcription. The arrow indicates the direction of transcription. The boxed sequences indicate a region that may function in polymerase binding and recognition. The inverted triangle is the site of the sequenced Tn*phoA* insertion junction. The arrow indicates a potential site for single sequence cleavage.

3 kilobases of DNA containing the *pagC* gene (from the *PstI* restriction endonuclease site 1500 nucleotides 5′ to the start of *pagC* translation to the *Eco*RI restriction endonuclease site 1585 nucleotides downstream of *pagC* translation termination) were inserted into the pJM703.1 derivative discussed above. The *pagC* sequence from the *Cla*I restriction endonuclease site was deleted (490 nucleotides) and replaced with a synthetic oligonucleotide polylinker that creates unique restriction endonuclease sites. DNA encoding one or more heterologous proteins, e.g., an antigen, can be inserted into this site. This creates a vector which allows the insertion of multiple foreign genes into the DNA surrounding *pagC*.

The vector can be mobilized into *Salmonella* by mating or any other delivery system, e.g., heat shock, bacteriophage transduction or electroporation. Since it can not replicate, the vector can only insert into *Salmonella* by site specific recombination with the homologous DNA on both sides of the *pagC* gene. This will disrupt and inactivate the native *pagC* locus and replace it with the disrupted *pagC* DNA carried on the vector.

Such recombination events can be identified by marker exchange and selective media if the foreign DNA inserted into the *pagC* locus confers a growth advantage. The insertion of antibiotic resistance genes for selection is less desirable as this could allow an increase in antibiotic resistance in the natural population of bacteria. Genes which confer resistance to substances other than antibiotics e.g., to heavy metals or arsenic (for mercury resistance, see Nucifora et al., 1989, J. Bact., 171:4241-4247, hereby incorporated by reference), can be used to identify transformants. Alternatively, selection can be performed using a *Salmonella* recipient strain that carries an auxotrophic mutation in a metabolic pathway and a vector that carries DNA that complements the auxotrophic mutation. Many *Salmonella* live vaccine prototypes contain mutations in histidine or purine pathways thus complementation of these metabolic auxotrophies can be used to select for integrants. (Purine mutations specifically have been shown to be too attenuated for use in man.) Further proof of marker exchange can be documented by loss of the ampicillin resistance (carried on the plasmid backbone) or by blot hybridization analysis.

A gene useful for selection can be cloned by complementation of a vaccine strain with a metabolic auxotrophy. Specific examples include the cloning of the DNA encoding both *purB* and *phoP* by complementation of a strain deleted for function of both these genes. *Salmonella* gene libraries have been constructed in a pLAFR cosmid vector (Frindberg et al., 1984, Anal. Biochem. 137:266-267, hereby incorporated by reference) by methods known to those skilled in the art. pLAFR cosmids are broad host range plasmids which can be mobilized into *Salmonella* from *E. coli*. An entire bank of such strains can be mobilized into *Salmonella* vaccine

strains and selected for complementation of an auxotrophic defect (e.g., in the case of *purB* growth on media without adenine). The DNA able to complement this defect is then identified and can be cloned into the antigen delivery vector.

As discussed above heterologous genes can be inserted into the polylinker that is inserted into the *pagC* sequence of the vector. The heterologous genes can be under the control of any of numerous environmentally regulated promotor systems which can be expressed in the host and shut off in the laboratory. Because the expression of foreign proteins, especially membrane proteins (as are most important antigens), is frequently toxic to the bacterium, the use of environmentally regulated promoters that would be expressed in mammalian tissues at high levels but which could be grown in the laboratory without expression of heterologous antigens would be very desirable. Additionally, high expression of antigens in host tissues may result in increased attenuation of the organism by diverting the metabolic fuel of the organism to the synthesis of heterologous proteins. If foreign antigens are specifically expressed in host phagocytic cells this may increase the immune response to these proteins as these are the cells responsible for processing antigens.

The promoter systems likely to be useful include those nutritionally regulated promoter systems for which it has been demonstrated that a specific nutrient is not available to bacteria in mammalian hosts. Purines, Sigwart et al., 1989, Infect. Immun., 57:1858 and iron, Finklestein et al., 1983, Rev. Infect. Dis. 5:S759, e.g., are not available within the host. Promoters that are iron regulated, such as the aerobactin gene promoter, as well as promoters for biosynthetic genes in purine pathways, are thus excellent candidates for testing as

promoters that can be shut down by growth in high concentrations of these nutrients. Other useful environmentally regulated *Salmonella* promoters include promoters for genes which encode proteins which are specifically expressed within macrophages, e.g., the DnaK and GroEL proteins, which are increased by growth at high temperature, as well as some *phoP* activated gene products, Buchmeier et al., 1990, Science 248:730, hereby incorporated by reference. Therefore, promoters such as the *pag*C 5′ controlling sequences and the better characterized promoters for heat shock genes, e.g., GroEL and DnaK, will be expected to be activated specifically within the macrophage. The macrophage is the site of antigen processing and the expression of heat shock genes in macrophages and the wide conservation of heat shock genes in nature may explain the immunodominance of these proteins. A consensus heat shock promoter sequence is known and can be used in the vectors (Cowling et al., 1985, Proc. Natl. Acad. Sci. USA 82:2679, hereby incorporated by reference).

The vectors can include an environmentally regulated T7 polymerase amplification system to express heterologous proteins. For example, the T7 polymerase gene (cloned by Stan Tabor and Charles Richardson, See Current Protocols in Molecular Biology ed. Ausubel et al., 1989, (page 3.5.1.2) John Wiley and Sons, hereby incorporated by reference) under control of an iron regulated promoter, can be included on the vectors described above. We have inserted the aerobactin gene promoter of *E. coli* with the sequence CATTTCTCATTGATAATGAGAATCATTATTGACATAATTGTTATTATTTTACG (SEQ ID NO:2), Delorenzo et al. J. Bact. 169:2624, hereby incorporated by reference, in front of the T7 polymerase gene and demonstrated iron regulation of the gene product. This version of the vector will also include

one or more heterologous antigens under the control of T7 polymerase promoters. It is well known that RNA can be synthesized from synthetic oligonucleotide T7 promoters and purified T7 in vitro. When the organism encounters low iron T7 polymerase will be synthesized and high expression of genes with T7 promoters will be facilitated.

pagC-fusion proteins in *S. typhimurium*

Expression of heterologous antigens within macrophages under the control of *phoP* regulated promotors can be used as an effective method of both attenuating *Salmonellae* and enhancing immunogenicity of foreign antigens. As discussed above, the expression of PagC is induced in antigen processing cell, i.e., a macrophage. Thus, expression of a heterologous antigen under the control of the *pagC* promoter is also likely to be inducible in macrophages.

To evaluate the immune respone to a heterologous antigen expressed under the control of inducible *pag* promoters, mice were inoculated with bacteria which expressed the antigen, AP, under the control of the *pagC* or *pagD* regulatory sequences. Pag-AP fusion proteins were produced in these bacteria from a single chromosomal copy of the gene encoding AP. The bacteria were generated utilizing two methods: Tn*phoA* mutagenesis, and genetic engineering techniques using a suicide vector, both of which are described above.

As a control, mice were innoculated with bacteria which expressed an AP fusion protein under the control of constitutive promoters. The constitutive promoter was completely independent of regulation by genes in the PhoP regulon. Two such strains of bacteria, Strain 610 and Strain 617, were constructed using methods described above. AP expression in Strain 610 was moderate, whereas AP expression in Strain 617 was high (see Fig. 14C).

These strains were injected intraperitoneally into BABL/C mice. Serum samples were taken three weeks after inoculation. Normal mouse serum (MNS) was used as a control. Standard ELISA assays were used to test the sera for the presence of AP-specific antibodies. Sera was also tested for LPS-specific antibodies using *S. typhimurium* LPS. Antibodies directed to LPS were detected in all the murine sera tested, but only those strains in which AP was expressed as a Pag fusion protein from a single chromosomal gene copy engendered an immune response against the model heterologous antigen, AP (see Figs. 14A and Fig. 14B).

Despite approximately 10-fold higher constitutive expression of the AP fusion in strain 617, only a minimal immune response to this antigen was noted after immunization with strain 617. In contrast, a strong response was observed in mice inoculated with strains which expressed the Pag-AP fusion protein. These data indicate that *phoP*-regulation which results in *in vivo* induction of protein expression within macrophages increases the immunogenicity of heterologous antigens expressed under the control of the *pag* promoters. Any promoter which directs cell-specific, inducible expression of a protein in macrophages or other antigen presenting cells, e.g., *pag* described herein, can be used to increase the immunogencity of an antigen expressed in *Salmonella*.

The *pagC* gene and *pagC* Gene Product
Strains, materials, and methods

The following strains, materials, and methods were used in the cloning of *pagC* and in the analysis of the gene and its gene product.

Rich media was Luria broth (LB) and minimal media was M9, Davis et al., 1980, supra. The construction of *S. typhimurium* strain CS119 *pagC1::TnphoA phoN2* zxx::6251 Tn*10d-Cam* was previously described, Miller et al., 1989,

supra. American Type Culture Collection (ATCC) *S. typhimurium* strain 10428 included CS018 which is isogenic to CS119 except for *phoP105*::Tn*10d*, Miller et al., 1989, supra, CS022 *pho-24*, Miller et al., 1990, J. Bacteriol. 172:2485-2490, hereby incorporated by reference, and CS015 *phoP102*::Tn10d-cam, Miller et al., 1989, supra. Other wild type strains used for preparation of chromosomal DNA included *S. typhimurium* LT2 (ATCC 15277), *S. typhimurium* Q1 and *S. drypool* (Dr. J. Peterson U. Texas Medical Branch, Galveston), and *Salmonella typhi* Ty2 (Dr. Caroline Hardegree, Food and Drug Administration). pLAFR cosmids were mobilized from *E. coli* to *S. typhimurium* using the *E. coli* strain MM294 containing pRK2013, Friedman et al., 1982, Gene 18:289-296, hereby incorporated by reference. AP activity was screened on solid media using the chromogenic phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (XP). AP assays were performed as previously described, Brickman et al., 1975, J. Mol. Biol. 96:307-316, hereby incorporated by reference, and are reported in units as defined by Miller, Miller, 1972, supra, pp. 352-355.

One dimensional protein gel electrophoresis was performed by the method of Laemmli, 1970, Nature, 227:680-685, hereby incorporated by reference, and blot hybridization using antibody to AP was performed as previously described, Peterson et al., 1988, Infect. Immun. 56:2822-2829, hereby incorporated by reference. Whole cell protein extracts were prepared, from saturated cultures grown in LB at 37°C with aeration, by boiling the cells in SDS-*pag*E sample buffer, Laemmli, 1970, supra. Two dimensional gel electrophoresis was performed by the method of O'Farrell, 1975, J. Biol. Chem. 250:4007, hereby incorporated by reference. Proteins in the 10% polyacrylamide slab gels were visualized by silver staining, Merril et al., 1984, Methods in

Enzymology, 104:441, hereby incorporated by reference.

Chromosomal DNA was prepared by the method of Mekalanos, 1983, Cell, 35:253-263, hereby incorporated by reference. DNA, size fractionated in agarose gels, was transferred to nitrocellulose (for blot hybridization) by the method of Southern, 1975, J. Mol. Biol. 98:503-517, hereby incorporated by reference. DNA probes for Southern hybridization analysis were radiolabeled by the random primer method, Frinberg et al., 1984, supra. Plasmid DNA was transformed into *E. coli* and *Salmonella* by calcium chloride and heart shock, Mekalanos, 1983, supra, or by electroporation using a Genepulser apparatus (Biorad, Richmond, Ca.) as recommended by the manufacturer, Dower et al., 1988, Nucl. Acids Res. 16:6127-6145, hereby incorporated by reference. DNA sequencing was performed by the dideoxy chain termination method of Sanger et al., 1977, Proc. Natl. Acad. Sci. USA, 74:5463-5467, hereby incorporated by reference, as modified for use with SEQUENASE® (U.S. Biochemical, Cleveland, Ohio). Oligonucleotides were synthesized on an Applied Biosystems Machine and used as primers for sequencing reactions and primer extension of RNA. Specific primers unique to the two ends of Tn*phoA*, one of which corresponds to the AP coding sequence and the other to the right IS50 sequence, were used to sequence the junctions of the transposon insertion.

Construction of a *S. typhimurium* cosmid gene bank in pLAFR3 and screening for clones containing the wild type *pag*C DNA was performed as follows. DNA from *S. typhimurium* strain ATCC 10428 was partially digested using the restriction endonuclease Sau3A and then size selected on 10-40% sucrose density gradient. T4 DNA ligase was used to ligate chromosomal DNA of size 20-30 kilobases into the cosmid vector pLAFR3, a derivative of pLAFR1, Friedman et al., 1982, Gene 18:289-296, hereby

incorporated by reference, that was digested with the restriction endonuclease *Bam*HI. Cosmid DNA was packaged and transfected into *E. coli* strain DH5-α using extracts purchased from Stratagene, La Jolla, Ca. Colonies were screened by blot hybridization analysis.

The analysis of proteins produced from cloned DNA by in vitro transcription/translation assays was analyzed as follows. These assays were performed with cell free extracts, (Amersham, Arlington Heights, Illinois), and were performed using conditions as described by the manufacturer. The resultant radiolabeled proteins were analyzed by SDS-*pag*E.

RNA was purified from early log and stationary phase *Salmonella* cultures by the hot phenol method, Case et al., 1988, Gene 72:219-236, hereby incorporated by reference, and run in agarose-formaldehyde gels for blot hybridization analysis, Thomas, 1980, Proc. Natl. Acad. Sci. USA 77:5201, hereby incorporated by reference. Primer extension analysis of RNA was performed as previously described, Miller et al., 1986, Nuc. Acids. Res. 14:7341-7360, hereby incorporated by reference, using AMV reverse transcriptase (Promega, Madison, Wisconsin) and synthesized oligonucleotide primers complementary to nucleotides 335-350 and 550-565 of the *pag*C locus.

Identification of an 18 kDa protein missing in a *pagC* mutant of *S. typhimurium*

*pag*C mutant strain CS119 was analyzed by two dimensional protein electrophoresis to detect protein species that might be absent as a result of the Tn*phoA* insertion. Only a single missing protein species, of approximately 18 kD and pI-8.0, was observed when strains, isogenic except for their transposon insertions, were subjected to this analysis. This 18 kDa species was also missing in similar analysis of *Salmonella* strains with mutations *phoP* and *phoQ*. Though two-dimensional

protein gel analysis might not detect subtle changes of protein expression in strain CS119, this suggested that a single major protein species was absent as a result of the *pagC::TnphoA* insertion.

Additional examination of the 2-dimensional gel analysis revealed a new protein species of about 45 kDa that is likely the *pagC*-Ap fusion protein. The *pagC*-AP fusion protein was also analyzed by Western blot analysis using antisera to AP and found to be similar in size to native AP (45 kDa) and not expressed in *PhoP-S. typhimurium*.

Cloning of the *pagC::TnphoA* insertion

Chromosomal DNA was prepared from *S. typhimurium* strain CS119 and a rough physical map of the restriction endonuclease sites in the region of the *pagC::TnphoA* fusion was determined by using a DNA fragment of *TnphoA* as a probe in blot hybridization analysis. This work indicated that digestion with the restriction endonuclease *eco*RV yielded a single DNA fragment that included the *pagC::TnphoA* insertion in addition to several kilobases of flanking DNA. Chromosomal DNA from strain CS119 was digested with *Eco*RV (blunt end) and ligated into the bacterial plasmid vector pUC19 (New England Biolabs) that had been digested with the restriction endonuclease *Sma*I (blunt end). This DNA was electroporated into the *E. coli* strain DH5-α (BRL) and colonies were plated onto LB agar containing the antibiotics kanamycin (*TnphoA* encoded and ampicillin (pUC19 encoded). A single ampicillin and kanamycin resistant clone containing a plasmid designated pSM100 was selected for further study.

A radiolabeled DNA probe from pSM100 was constructed and used in Southern hybridization analysis of strain CS119 and its wild type parent ATCC 10428 to prove that the *pagC::TnphoA* fusion had been cloned. The

probe contained sequences immediately adjacent to the transposon at the opposite end of the AP gene [*Hpa*I endonuclease generated DNA fragment that included 186 bases of the right IS50 of the transposon and 1278 bases of *Salmonella* DNA (Fig. 2). As expected, the pSM100 derived probe hybridized to an 11-12 kb *AccI* endonuclease digested DNA fragment from the strain containing the transposon insertion, CS119. This was approximately 7.7kb (size of Tn*phoA*) larger than the 3.9 kB *AccI* fragment present in the wild type strain that hybridizes to the probe. In addition, a derivative of plasmid pSM100, pSM101 (which did not allow expression of the *pagC*-PhoA gene fusion off the *lac* promoter), was transformed into *phoP*- (strain Cs015) and *phoN*- (strain CS019) *Salmonella* strains and the cloned AP activity was found to be dependent on *phoP* for expression. Therefore we concluded that the cloned DNA contained the *pagC*::Tn*phoA* fusion.

The presence of the *pagC* gene was also demonstrated in other strains of *S. typhimurium*, as well as in *S. typhi*, and *S. drypool*. All *Salmonella* strains examined demonstrated similar strong hybridization to an 8.0 kb *Eco*RV and a 3.9 kb *Acc*I restriction endonuclease fragment suggesting that *pagC* is a virulence gene common to *Salmonella* species.

The *pagC* gene probe from nucleotides -46 (with 1 as the first base of the methionine to 802 (*Pst*I site to the *Bgl*II site) failed to cross hybridize to DNA from *Citrobacter freundii, Shigella flexneri, Shigella sonnei, Shigella dysenterial, Escherichia coli, Vibrio cholerae, Vibrio vulnificus, Yersenia entero colitica, and Klebsiella pneumonia.*

Cloning of the wild type *pagC* locus DNA and its complementation of the virulence defect of a *S. typhimurium* pagC mutant

The same restriction endonuclease fragment described above was used to screen a cosmid gene bank of wild type strain ATCC 10428. A single clone, designated pWP061, contained 18 kilobases of *S. typhimurium* DNA and hybridized strongly to the *pagC* DNA probe. pWP061 was found to contain *Salmonella* DNA identical to that of pSM100 when analyzed by restriction endonuclease analysis and DNA blot hybridization studies. Probes derived from pWP061 were also used in blot hybridization analysis with DNA from wild type and CS119 *S. typhimurium*. Identical hybridization patterns were observed to those seen with pSM100. pWP061 was also mobilized into strain CS119, a *pagC* mutant strain. The resulting strain had wild type virulence for BALB/c mice (a LD_{50} less than 20 organisms when administered by IP injection). Therefore the cloned DNA complements the virulence defect of a *pagC* mutant strain.

Since, a wild type cosmid containing *pagC* locus DNA was found to complement the virulence defect of a *pagC* mutant *S. typhimurium* strain, it was concluded that the *pagC* protein is an 188 amino acid (18 kDa) membrane (see below) protein essential for survival within macrophages and virulence of *S. typhimurium*.

Physical mapping of restriction endonuclease sites, DNA sequencing, and determination of the *pagC* gene product

Restriction endonuclease analysis of plasmid pSM100 and pWP061 was performed to obtain a physical map of the *pagC* locus, and, in the case of PSM100, to determine the direction of transcription (Fig. 2). DNA subclones were generated and the Tn*phoA* fusion junctions were sequenced, as well as the *Salmonella* DNA extending from the *Hpa*I site, 828 nucleotides 5′ to the *phoA* fusion junction, to the *Eco*RI site 1032 nucleotides 3′ to the

Tn*phoA* insertion (Fig. 2 and 3). The correct reading frame of the DNA sequence was deduced from that required to synthesize an active AP gene fusion. The deduced amino acid sequence of this open reading frame was predicted to encode a 188 amino acid protein with a predicted pI+8.2. This data were consistent with the 2-D polyacrylamide gel analysis of strain CS119 in which an 18 kDa protein of approximate pI+8.0 was absent. No other open reading frames, predicted to encode peptides larger than 30 amino acids, were found.

The deduced amino acid sequence of the 188 amino acid open reading frame contains a methionine start codon 33 amino acids from the fusion of *pag*C and AP (Fig. 3). This 33 amino acid *pag*C contribution to the fusion protein was consistent with the size observed in Western blot analysis and contains a hydrophobic N-terminal region, identified by the method of Kyle et al., 1982, J. Mol. Biol. 157:105-132, hereby incorporated by reference, that is a typical bacterial signal sequence, Von Heinje, 1985, J. Mol. Biol. 184:99-105, hereby incorporated by reference. Specifically, amino acid 2 is a positively charged lysine, followed by a hydrophobic domain and amino acid 24 is a negatively charged aspartate residue. A consensus cleavage site for this leader peptide is predicted to be at an alanine residue at amino acid 23, Von Heinje, 1984, J. Mol. Biol. 173:243-251, hereby incorporated by reference. The DNA sequence also revealed a typical ribosomal binding site, Shine et al., 1974, Proc. Natl. Acad. Sci. USA 71:1342-1346, hereby incorporated by reference, at 6-2 nucleotides 5′ to the predicted start of translation (Fig. 3) nucleotides 717-723). This suggested that the open reading frame was, in fact, translated and further supported the assumption that this was the deduced amino acid sequence of the *pag*C protein interrupted by the Tn*phoA* insertion (Fig. 3).

In vitro synthesis of proteins by the cloned *pagC* locus

To detect if other proteins were encoded by *pagC* and to determine the approximate size of the *pagC* gene product, an *in vitro* coupled transcription/translation analysis was performed. A 5.3 kilobase *Eco*RI fragment of pWP061 was inserted into pUC19 so that the *pagC* gene would not be expressed off the *lac* promotor. This plasmid was used in an *in vitro* coupled transcription-translation assay. A single protein of approximately 22 kilodaltons was synthesized by the cell free system. The size was compatible with this being the precursor of the *pagC* protein containing its leader peptide. These data further support the conclusion the single and the single *pagC* gene product had been identified.

Identification of the *pagC* encoded RNA

An approximately 1100 nucleotide RNA is encoded by *pagC*. The *pagC* gene is highly expressed by cells with a *phoP* constitutive phenotype of *pag* activation, as compared to wild type and *phoP* constitutive phenotype of *pag* activation, as compared to wild type and *phoP*$^-$ bacteria. In these blot hybridization experiments *pagC* is only detected in wild type cells grown in rich media during stationary growth. This result, coupled with previous work, Miller et al., 1989, supra, Miller et al., 1990, supra, demonstrates that *pagC* is transcriptionally regulated by the *phoP* gene products and is only expressed during early logarithmic phase growth in rich media by cells with a *phoP* constitutive phenotype.

The size of the *pagC* transcript is approximately 500 nucleotides greater than that necessary to encode the 188 amino acid protein. Primer extension analysis of *Salmonella* RNA using oligonucleotide primers specific for *pagC* sequence was performed to determine the approximate start site of transcription and to determine whether these nucleotides might be transcribed 5′ or 3′ to the

188 amino acid *pagC* gene product. Primer extension analysis with an oligonucleotide predicted to be complementary to nucleotides 550-565 of *pagC*, 150 nucleotides 5′ to the predicted start codon, resulted in an approximately 300 nucleotide primer extension product. Therefore a primer further upstream was constructed complementary to nucleotides 335-350 of *pagC* and used in a similar analysis. A primer extension product of 180 nucleotides was observed to be primer specific. This is consistent with transcription starting at nucleotide 170 (Fig. 3). Upstream of the predicted transcriptional start, at nucleotides 153-160, a classic RNA polymerase binding site was observed with the sequence TATAAT at -12 nucleotides as well as the sequence TAATAT at -10 nucleotides. No complete matches were observed for the consensus RNA polymerase recognition site (TTGACA) 15-21 nucleotides upstream from the -10 region. AT -39 (126-131) nucleotides (TTGGAA), -38 (127-132) nucleotides (TTGTGG), and -25 (135-140) nucleotides (TTGATT) are sequences that have matches with the most frequently conserved nucleotides of this sequence.

Based on the above results transcription was predicted to terminate near the translational stop codon of the 188 amino acid protein (nucleotide 1295, Fig. 3). Indeed, a stem loop configuration was found at nucleotides 1309-1330 that may function as a transcription terminator. This was consistent with the lack of evidence of open reading frames downstream of the 188 amino acid protein and the lack of synthesis of other transcription/translation using the cloned *pagC* DNA. This further suggests that the *pagC::TnphoA* insertion inactivated the synthesis of only a single protein.

Similarity of *pagC* to Ail and Lom

A computer analysis of protein similarity using the National Biomedical Research Foundation/Protein Identification Resource, George et al., 1986, Nucleic Acids Res. 14:11-15, hereby incorporated by reference, protein sequence base was conducted to identify other proteins that had similarity to *pagC* in an attempt to find clues to the molecular function of this protein. Remarkably, *pagC* was found to be similar to a bacteriophage lambda protein, Lom, that has been localized to the outer membrane in minicell analysis, Court et al., 1983, Lambda II, Hendrix, R.W. et al. ed. Cold Spring Harbor Laboratory (Cold Spring Harbor NY), pp. 251-277, hereby incorporated by reference, and demonstrated to be expressed by lambda lysogens of *E. coli*, Barondess, et al., 1990, Nature 346:871-874, hereby incorporated by reference. Recently, the deduced amino acid sequence of the cloned *ail* gene product of *Y. enterocolitica* was determined and found to also be similar to Lom, Miller et al., 1990b, J. Bacteriol. 172:1062-1069. Therefore, a protein family sequence alignment was performed using a computer algorithm that establishes protein sequence families and consensus sequences, Smith et al., 1990, Proc. Natl. Acad. Sci. 87:118-122, hereby incorporated by reference. The formation of this family is indicated by the internal data base values of similarity between these proteins : *pagC* and Lom (107.8), *pagC* and Ail (104.7), and Ail and Lom (89.8). These same proteins were searched against 314 control sequences in the data base and mean values and ranges were 39.3 (7.3-52.9) *pagC*, 37.4 (7.3-52.9) Ail, and 42.1 (7.0-61.9) Lom. The similarity values for this protein family are all greater than 3.5 standard deviations above the highest score obtained for similarity to the 314 random sequences. No other

similarities or other family members were found in the database. Regions of similarity are located not only in the leader peptide transmembrane domains but throughout the protein.

pag Mutant Strains Are Attenuated For Virulence

Salmonella typhimurium strains of the invention with a *pagC* mutation were attenuated for virulence by least 1,000-fold.

In addition *pagC*, other *pag* genes described herein may be useful in the development of live *Salmonella* vaccines. Mutations in *phoP*-activated genes could be used to construct attenuated, live *Salmonella* vaccines. In constructing multivalent *Salmonella* vectored vaccines, PhoP-activated promoters could increase immunogenecity by targeting foreign protein expression to antigen presenting macrophages.

Identification of novel *phoP*-activated genes.

To further analyze the role of *phoP*-activated genes in bacterial virulence, a bank of strains with active *phoA* gene fusions was generated by Tn*phoA* mutagenesis. Strain CS019 was the parent strain for Tn*phoA* mutagenesis because it has wild-type bacterial virulence and carries the *phoN2* allele, which results in minimal background phosphatase activity. Strains with active *phoA* gene fusions were identified by blue colony phenotype after growth in agar containing XP. Such strains were then screened for decreased fusion protein activity on acquisition of the *phoP12* allelle that results in a PhoP-null phenotype.

Two thousand and sixty-four AP expressing strains were isolated and colony purified from two hundred and forty independent matings. Strains with AP activity were isolated at a frequency of 0.8% from the total pool of kanamycin resistant (Tn*phoA* containing) bacteria. A total of fifty-four candidate *pag*::Tn*phoA* insertions were

isolated from the AP expressing strain bank, and forty-nine of these were determined to have greater than six-fold reduction in AP activity in the absence of functional *phoP/phoQ*. Therefore, approximately 2% of the colonies expressing AP were identified as *pag-phoA* gene fusions.

Identification of thirteen unique *pag* loci.

Three methods were used to determine whether the forty-nine Tn*phoA* insertions defined unique *pag* loci. First, physical maps of the *EcoRI* and *Hind*III restriction endonuclease sites 5′ to the Tn*phoA* insertions were defined. Second, linkage analysis to transposon insertions highly linked to known *pag* loci was performed. Third, strains determined to be unique by the above methods were screened for linkage to a bank of strains with transposon insertions of known chromosomal location.

Blot hybridization analysis demonstrated that thirteen of the forty-nine strains had unique restriction endonuclease sites 5′ to the Tn*phoA* insertion. The numbers of strains with similar physical maps 5′ to the Tn*phoA* insertion ranged from 1-7. One of the thirteen physical maps was similar to that expected for an insertion in *pagC* and was noted in seven of the strains isolated as containing candidate *pag*::Tn*phoA* insertions. Analysis of these seven strains indicated that only three of these were *pagC*::Tn*phoA* insertions, since blot hybridization analysis with a fragment of *pagC* as a probe and linkage analysis to transposon insertions highly linked to *pagC* indicated that four of these insertions were not in *pagC*. Another of the *pag*::*phoA* fusions, denoted *pagP*, had the physical 5′ restriction-endonuclease map that would be expected for *phoN*. However, this insertion was determined not to be within *phoN* by linkage analysis and blot hybridization. A transductional cross was performed between wild type

bacteria and strain CS1247 containing *pagP*::Tn*phoA* and zxx::6215Tn*10d-cam*. These transductants were selected on kanamycin, insuring the inheritance of the *pagP*::Tn*phoA* which encodes kanamycin resistance. These colonies were then screened for choramphenicol resistance which would indicate linkage of zxx:6215Tn*10d-cam* to *pagP*. No linkage was found indicating that *pagP* was not linked to *phoN*. Blot hybridization using a portion of *phoN* as a probe was also performed on CS1247 and indicated that this strain contained a wild type *phoN* locus. Thirteen *pag* loci were defined and designated *pagD-P*.

To further define the PhoP regulation of the 13 *pag*::Tn*phoA* fusion proteins, AP activity was assayed in strains isogenic except for the *phoP* locus. AP activity was assayed during bacterial growth in rich medium in logarithmic and stationary growth phase (Table 13). The dependence of an intact *phoP* locus for full expression remained constant for the different stages of growth; however, the relative amount of AP expression increased as growth was limited. The difference in expression of *pag* gene fusions varied from six to forty-eight fold when isogenic strains with a wild type and null *phoP* locus were compared.

Of the five previously identified *pag* loci, only *phoN*, *pagC*, and *pagA* have known chromosomal locations. Linkage analysis of the 13 newly identified *pag* loci was performed using strains containing transposon insertions linked to *pagC* (AK3233 and AK3140), and to *pagA* (AK3255). Three *pag*::Tn*phoA* insertions were found to be linked to AK3140 which is in a region near *pagC* at 24-25 minutes on the chromosome. These were designated *pagD*, *pagE*, and *pagF*. *PagD*::Tn*phoA* was similarly linked to the transposon insertion of AK3233 (83%) and AK3140 (33%) as was previously reported for *pagC*. The Tn*phoA* insertion of this strain has been further defined and is

divergently transcribed from *pagC*. *pagE* and *pagF* exhibited different linkage to the insertions of AK3233 and AK3140 than *pagC* and *pagD* suggesting a significantly different chromosomal location. The *pagE*::Tn*phoA* insertion is 39% linked to the transposon insertion of AK3233 and 99.1% linked to that of AK3140, while *pagF*::Tn*phoA* is 31% linked to the insertion of AK3140 but not to that of AK3233. These different linkages in addition to the physical maps of the restriction endonuclease sites 5′ to the Tn*phoA* insertion indicated that these were new *pag* loci. Therefore, three new *pag* loci were found in the region of 25 minutes, one of which is highly linked to the previously defined *pagC*.

Linkage analysis was then performed using a group of defined random Tn*10*Δ16Δ17 insertions on the ten strains with Tn*phoA* insertions of no known location. Of these ten *pag*::Tn*phoA* alleles only two demonstrated linkage to the bank of Tn*10*Δ16Δ17 insertions. The *pagG*::Tn*phoA* insertion was demonstrated to have 97% linkage to the transposon insertion of AK3258 located at approximately 30 minutes. The *pag*::Tn*phoA* insertion, designated *pagH*, exhibited 23% linkage to the insertion of AK3091. The linkage to the transposon insertion of AK3091 was similar to linkage previously demonstrated for *prgE* (26%). Therefore, this chromosomal region contains both PhoP-activated and repressed genes. This TnΔ16Δ17 insertion was analyzed using pulse field gradient electrophoresis of chomosomal DNA from AK3091 digested with the restriction endonuclease *XbaI* and *BlnI*. These data indicate that the transposon insertion of AK3091 was located in the region of 20-25 minutes and that *pagH* and *prgE* are located in this region of the chromosome.

Strains with *pag*:Tn*phoA* insertions have wild type sensitivity to the rabbit NP-1 defensin

S. Typhimurium strains with null mutations in the *phoP* operon have increased sensitivity to a variety of cationic antimicrobial peptides including defensins, magainins, and protamine. The defensins are a family of mammalian peptides present in the granules of neutrophils, lung macrophages, and intestinal Paneth cells. Resistance to these peptides may contribute to bacterial virulence and the ability to colonize mucosal surfaces. Strains with *pag*::Tn*phoA* insertions were tested for sensitivity to the highly active rabbit defensin NP-1. None of the strains with single *pag*::Tn*phoA* insertions demonstrated increased sensitivity to NP-1 defensin (see Fig. 6). Thus despite the demonstrated sensitivity of PhoP-null mutants to rabbit defensin NP-1, no defined mutations in *pag* loci were associated with sensitivity to defensins.

Four strains with *pag*::Tn*phoA* insertions demonstrate marked attenuation for mouse virulence

To further define whether these new *pag* loci contributed to mouse virulence, the 13 strains with *pag* transposon insertions were screened *in vivo*. Mice were injected intraperitoneally with approximately 100 organisms. Four strains with transposon insertions in *pagD*, *pagJ*, *pagK*, and *pagM* demonstrated attenuated virulence. Mice injected with these strains all survived and showed no signs of systemic infections, such as hepatosplenomegaly and scruffiness (piloerection due to fever). These four strains were subjected to further virulence testing by intraperitoneal injection of multiple doses of organisms in a total of ten mice on two separate occasions. The mean LD_{50} was determined from these subsequent injections and is listed in Table 14. One of these strains, containing the *pagD*::Tn*phoA* insertion, has a LD_{50} 10,000 fold greater than wild-type

S. typhimurium. The other three strains were also markedly attenuated for mouse virulence with LD_{50} values greater than 1000-10,000 times that of wild type organisms. These data indicated that the PhoP-regulated loci, pagD, pagJ, pagK, and pagM, when mutated, result in attenuation of bacterial virulence.

pag::Tn*phoA* strains attenuated for mouse virulence have reduced survival within macrophages.

Since PhoP mutant *Salmonella* are deficient in survival within macrophages, strains containing mutations in *pag* genes that had attenuated mouse virulence were tested for reduced viability within macrophages. As shown Table 14, all strains with *pag* mutations demonstrated significantly reduced survival within macrophages. Decreased intracellular survival of *pag* mutants was not observed until a time when *pag* are predicted to be maximally expressed.

Four strains with mutations in the *pagC*, *pagD*, *pagJ*, *pagK* and *pagM* loci were found to be attenuated for mouse virulence and survival within macrophages. Strains with mutations in these five *pag* all had varying degrees of virulence attenuation. Strains with a mutation in *pagJ* had a virulence defect comparable to that observed for *pagC* mutants (greater than 1000 x the LD_{50} of wild type organisms). The *pagD*::Tn*phoA* insertion resulted in the greatest attenuation of virulence, comparable to that of a PhoP null mutation (greater than 10,000 x the LD_{50} of wild type organisms). *pagK* and *pagM* mutants had virulence attenuation that was intermediate between the *pagJ* and *pagD* mutants. The cumulative effect of deletion of *pagC*, *pagD*, *pagJ*, *pagK*, and *pagM*, if additive and similar to the attenuation observed with Tn*phoA* insertions, may be much greater than that observed by deletion of *phoP* alone. The observation that many of these genes are somewhat expressed in stationary phase in the absence of PhoP suggests that functional Pag proteins

could be produced *in vivo* in the absence of PhoP. One virulence gene *pagM* is significantly expressed in the absence of PhoP, though it may still require PhoP/PhoQ for induction within macrophage phagosomes. This data suggests that deletion of *pag* gene products could lead to greater virulence attenuation than deletion of the regulatory proteins.

Salmonella envelope proteins as virulence factors: Defensin senstivity

Based on the methods used to identify *pag* loci, i.e., translational gene fusions to *phoA*, and the observation that the pagC gene fusions produce AP, it has now been discovered that many *pag* encode bacterial envelope proteins. No strains have been found with single *pag* mutations that confer sensitivity to defensins or other cationic peptides. The data suggest that an alteration of the bacterial envelope as a result of the change in synthesis of the entire aggregate of envelope proteins mediated by PhoP/PhoQ may be important to *S. typhimurium* virulence.

Defensins are small amphipathic cationic peptides of approximately 30-35 amino acids in length whose anti-microbial action involves penetration and disruption of membranes, possibly by forming selective anionic channels. Though defensins are largely found in neutrophils and Paneth cells these or other related molecules likely contribute to non-oxidative killing of phagocytosed bacteria by macrophages. Though it remains possible that a single unidentified *pag* encodes a protein responsible for defensin resistance, it seems more likely that a cumulative effect of expression of several *pag* encoded envelope proteins could result in resistance to defensins. An aggregate change in a large number of bacterial envelope proteins could alter the membrane charge, electrical potential, or lipid content such that defensin interaction with bacterial membranes could be

changed.

Identification of transcriptional units linked to *pagC*.

To identify genes upstream of *pagC*, *E. coli* carrying plasmid pWPL17 containing 2.8kb of DNA 5′ to *pagC* (Table 15 and Fig. 7) was mutagenized with the transposons Mud*J* and Tn*phoA*, and strains with AP or β-galactosidase activity were identified on chromogenic substrates. In addition, as part of an effort to identify additional PhoP-activated genes, random mutagenesis of the *Salmonella* chromosome with Tn*phoA* was performed, and strains with AP activity were screened for Tn*phoA* insertions linked to the Tn*10Δ16Δ17* of strain AK3233, which is 75% linked to *pagC*. Several strains that contained plasmids with active Mud*J* or Tn*phoA* generated gene fusions were identified. In addition, two strains were identified that contained active chromosomal Tn*phoA* insertions closely linked to *pagC*. Physical maps of the restriction endonuclease sites surrounding the transposon insertions in strains with active plasmid or chromosomal *lacZ* and *phoA* gene fusions were performed to determine the relationship of the transposon insertions to *pagC*. This analysis revealed that several regions of the DNA were transcribed oppositely to *pagC* (Fig. 7). Several Tn*phoA* insertions that resulted in active *phoA* gene fusions were identified. These data indicated that *pagC*-linked genes encoded membrane or secreted proteins.

Genes linked to *pagC* encode four novel proteins.

To further analyze the genes defined by transposon insertions, the DNA sequence of this region was determined (Fig. 8). DNA containing this region was cloned; 4 kb of DNA between the *Hpa*I site 737bp upstream of the start codon of *pagC* to a *Cla*I site far upstream was sequenced. The DNA sequence of the fusion junctions of all Tn*phoA* and Mud*J* gene fusions was also determined. Based on these data, the correct reading frame of each

gene was determined. The DNA sequence data revealed four ORFs predicted to be transcribed and translated based on the data derived from the Tn*phoA* and Mu*dJ* insertions. All ORFs revealed typical ribosome binding sites 6 to 11 bases from the predicted start of translation. The translation of the ORF immediately upstream and oppositely transcribed to *pagC*, *pagD*, indicates that a short envelope protein of 87 amino acids (unprocessed) is encoded. It is followed by a second ORF (*envE*) which encodes an envelope protein of 178 amino acids (unprocessed). This ORF is followed by a structure that could function as a Rho-independent transcriptional terminator (see Fig. 8). The third ORF, *msgA* (macrophage survival gene), encodes a small protein similar in size to that of the first gene product (79 amino acids) and is also followed by a structure that could function as a Rho-independent transcriptional terminator (see Fig. 8). The DNA sequence predicts that this protein is composed of several charged residues with a large number of negatively charged amino acids residing at the carboxy terminus. The predicted protein product does not contain a structure resembling a signal sequence at its amino terminus nor any hydrophobic stretches; therefore, the third ORF is unlikely to encode an envelope protein. The final ORF (*envF*) encodes an envelope protein of 278 amino acids (unprocessed). A computer search of known protein motifs revealed that EnvF contains a consensus prokaryotic membrane lipid attachment site and, therefore, is likely to be a lipoprotein (see Fig. 8 for consensus site location).

The predicted proteins produced by *pagD*, *envE*, and *envF* contain a typical bacterial signal sequence structure. In addition, hydrophobic profiles confirmed the hydrophobic nature of the amino-termini of these proteins. The EnvE and EnvF proteins also contain

hydrophobic stretches that could function as membrane spanning domains. The G+C content of the genes in this region are: *pagC*, 43.4%; *pagD*, 42.1%; *envE*, 45.9%; *msgA*, 46.8%; and *envF*, 40.5%, which is considerably lower than the average G+C content of *S. typhimurium* (52%). A complete search of the database with the predicted protein sequences of these four ORFs showed no significant similarities. Strains containing three distinct Tn*phoA* insertions and one Mud*J* insertion, each located in one of the four genes, were chosen for further characterization.

A gene *pagD*, oppositely transcribed to *pagC*, is positively regulated by PhoP/PhoQ

Representative strains with transposon insertions were examined to evaluate whether genes transcribed oppositely to *pagC* were increased in synthesis in the presence of PhoP. To accurately determine if these genes were PhoP regulated, it was necessary to recombine plasmid insertions onto the *Salmonella* chromosome. Upon replacement of the wildtype gene with the gene containing the transposon insertion, P22HT*int* lysates made on these strains were transduced into a PhoP deleted ($PhoP^-$) strain and AP or β-galactosidase levels were monitored. One of these transposon generated gene fusions demonstrated a significant increase in activity between $PhoP^-$ and WT backgrounds, while the other insertions showed no PhoP regulation (Table 16). This *pagD* loci is adjacent to and divergently transcribed from *pagC*.

The representative transposon insertion in *envF* was unable to be recombined onto the chromosome, likely due to an insufficient amount of homologous DNA downstream of the transposon. In order to examine the possibility of PhoP regulation of the *envF* gene, a region upstream of this gene through and including the *phoA* gene of the Tn*phoA* transposon was cloned as a 3-kb *Pvu*I (blunt-ended)-*Xho*I fragment into the *Eco*RV-*Sal*I sites of

the suicide vector pGP704. This clone was mated into *Salmonella* strain CS019, and ampicillin-resistant recombinants were selected (creating a strain designated *envF*::pGPP2). A *phoP*105::Tn10d-Tet mutation was transduced into this strain to create an isogenic pair differing only in the ability to produce a functional PhoP protein. As shown in Table 16, the introduction of the *phoP*105::Tn10d-Tet had no effect on the AP levels of these two strains, demonstrating that *envF* is not a PhoP-activated gene.

Transposon insertions in *pagC*-linked genes attenuate virulence and cause reduced survival within macrophages

Since transposon insertions in *pagC* significantly increase the LD_{50} of *S. typhimurium* in BALB/c mice, strains containing transposon insertions linked to *pagC* were evaulated for attenuation of mouse virulence. As shown in Fig. 7, while the transposon insertion in *envE* had no affect on strain virulence, a Tn*phoA* insertion in *pagD* and the Mud*J* insertion 1.8 kb downstream in *msgA* attenuate *S. typhimurium* virulence by greater than 300 fold as compared to wild-type organisms (LD_{50}<20 organisms). These data suggested that these two loci are essential to virulence.

To examine the survival capabilities of those strains having a virulence defect, *S. typhimurium* containing insertions in either *pagD* or *msgA* were used to infect bone marrow-derived macrophages. The results, shown in Table 15, demonstrate a macrophage survival defect for these two strains. The survival defect is greater for the *pagD* insertion (MSI=0.002) compared with the *msgA* insertion (MSI=0.01), and both defects are equal to or greater than that of the PhoP$^-$strain (MSI=0.01).

Transposon insertions in this gene could not be recombined onto the chromosome. Thus, it was necessary to demonstrate that the virulence and macrophage survival defects of *msgA* was not due to a polar effect of the Mud*J*

insertion on *envF* transcription. Therefore, pGPP2 was recombined into the *msgA*::Mud*J* strain and AP activity of this strain was compared to that of CSO19 containing the recombinant pGPP2. This data (shown in Table 16) demonstrates that the transcription of the *envF* gene is unaffected by the *msgA*::Mud*J* insertion and is transcribed from its own promoter. However, it is possible that under different environmental conditions, other promoters may be activated that could place *msgA* and *envF* on the same transcript.

Determination of the *msgA* and *pagD* transcriptional start sites

The 5′ regions of these genes were examined to define the transcriptional start sites of *msgA* and *pagD*. Oliogonucleotides complimentary to the 5′ end of each ORF or upstream region were used in a primer extension analysis. The results of this analysis revealed that the *pagD* transcript begins 39 bases upstream of the translational start. The predicted -10 (TTCCAT) and -35 (TTGAAT) regions were found to be similar to the known consensus sequences for *E. coli* promoters. The *pagD* transcript was detected only in PhoP^c *Salmonella* RNA and not in RNA from PhoP$^-$ *Salmonella*. The *msgA* transcriptional start was found to begin 58 bases upstream of the translational start and contain predicted -10 (CAAAAC) and -35 (TTACGT) sequences. These regions do not conform well to consensus -10 and -35 sequences; however, the cDNA from this transcript was easily detected using various primers in primer extensions of both PhoP^c and PhoP$^-$ RNA and appears to produce an abundant RNA.

Distribution of *pagD* and *msgA* genes in the Enterobacteriaceae and in two G+C content organisms

The G+C content of the *pagC* chromosomal region is much lower than the average G+C content of *Salmonella*. The gene encoding the PhoP-regulated acid phosphatase of

S. typhimurium (phoN) also has a low G+C content (39%), and DNA homologous to *phoN* was found only in two low G+C organisms of several genera tested. The DNAs of several members of the Enterobacteriaceae and two low G+C organisms were examined for similarity to *pagD* and *msgA* by blot hybridization. PCR fragments highly specific to each ORF were labeled and used as probes. This analysis demonstrated hybridization at high stringency to all *Salmonella* species examined as well as *Shigella sonnei, Shigella flexneri, Klebsiella pneumoniae* and *Citrobacter freundii*. No hybridization was seen to the low G+C organisms *Morganella morganii* or *Providencia stuartii*. Identical hybridization patterns were seen with probes specific for both genes indicating that these genes are also linked in organisms other than *Salmonella*.

A virulence gene cluster required for *Salmonella typhimurium* survival within macrophage macrophages

Four genes upstream and oppositely transcribed to the *pagC* gene of *Salmonella typhimurium* have now been identified. Three genes (*pagD, envE* and *envF*) are predicted to be envelope proteins based on the isolation of active Tn*phoA* insertions in these loci and the presence of a typical signal sequence at the amino-terminus of each protein. None of the four proteins possess significant homology to any protein in the database.

Only the gene immediately upstream of *pagC* and oppositely transcribed (*pagD*) was determined to be PhoP regulated. Transposon insertions in this gene greatly attenuate virulence and the ability of the organism to survive within murine macrophages. The transcription of several *pag* (including *pagC*) has been shown to be induced when *Salmonella* are within macrophage phagosome. In addition, analysis of proteins produced by *Salmonella* after infection of macrophage-derived cell lines indicate that *pag* products are induced and that *pagC* may be among

the most abundant gene products induced upon macrophage infection. Since *pagD* is required for macrophage survival, it is likely that the transcription of this gene also will be induced within macrophage phagosomes. The *pagD* protein is small (87 amino acids, unprocessed) and has no strong hydrophobic domains; therefore, it is likely that it is a periplasmic or secreted protein.

Transposon insertions in the *msgA* gene were found to have an effect on mouse virulence and macrophage survival. It is likely that this gene may also be induced within acidified macrophage phagosomes as are other genes necessary for macrophage survival. If this gene is induced by the macrophage environment, its expression (as well as other genes necessary for macrophage survival) may be controlled by a regulatory system separate from the PhoP/PhoQ system.

These *pagC*-linked genes do not appear to form an operon. Because none of the genes downstream of *pagD* are PhoP regulated, they appear not be transcribed from the *pagD* promoter. The presence of a potential transcriptional terminator at the end of the *envE* gene makes it unlikely that *msgA* is co-transcribed with *envE*. The data suggest that the *msgA*::Mud*J* insertion is not polar on *envF*, which suggests that *envF* has its own promoter. Additionally, a potential transcriptional terminator following *msgA* as well as a 493 bp intergenic region makes it unlikely that these genes are co-transcribed. Primer extension analysis of these genes confirms that all four genes are transcribed from their own promoter.

The other two genes identified in this region, *envE* and *envF*, appear to produce membrane proteins that contain characteristic membrane spanning regions. The *envF* gene product is likely to be a lipoprotein based on the presence of a consensus lipid attachment site and is

likely to play a role in *Salmonella* virulence.

The low G+C content of the genes in the *pagC* region suggests that they may have been acquired by horizontal transmission. Southern blot analysis of low G+C organisms probed with the *msgA* or *pagD* genes showed no homology, but this does not eliminate the possibility that they were acquired from another low G+C content organism. The possibility also exists that these genes reside on a mobile genetic element acquired from another source. The *msgA* and *pagD* probes hybridized in identical patterns to some members of the Enterobacteriaceae other than *Salmonella*. However, the *pagC* gene has been shown to be unique to *Salmonella* species. This may indicate that the products of the genes upstream of *pagC* do not form a complex with PagC or that their functions do not require PagC interaction. Alternatively, because proteins that have homology to PagC exist in other Enterobacteriaceae (in the absence of any DNA homology), a PagC homolog may be linked to *msgA* and *pagD* in other species which was not detected by the DNA hybridization experiments.

pagC/pagD promoter region: expression of heterologous proteins

pagC and *pagD* are divergently transcribed and are both PhoP activated. Other divergently transcribed, regulated genes are known in the art (Beck et al., 1988, Microbiol. Rev. 52:318-326), e.g., the *Klebsiella pneumoniae pulA-malX* region (Chapon et al., 1985, J. Bacteriol. 164:639-645). Transcription of most of such genes require accessory proteins, such as CAP, in addition to the regulator to activate transcription. These two genes are divergently transcribed, and their promoters are arranged back-to-back. A region of 134 bp exists between transcriptional start sites of these genes, which is similar to the intergenic region between *pagC* and *pagD*. The *pulA-malK* promoter region is

predicted to contain two MalT (the regulatory protein of this system) binding sites, one for each gene. Other MalT-activated genes require the CAP protein for expression, but the *pulA* and *malX* genes do not, possibly because of the high local concentration of the MalT regulator. Since the region between the transcriptional start sites of *pagC* and *pagD* (the predicted -35 sequences) is only 137bp (nucleotides 562 to 776 of SEQ ID NO:15), it is likely that only PhoP binding sites exist in the intergenic region, and that binding of one or more phosphorylated PhoP molecules positively regulates both genes. This *pagC/pagD* intergenic region which contains the divergent promoters can be used to construct vectors to express two heterologous proteins, one in each direction.

prg genes

As discussed above, *phoP/phoQ* constitutive mutations (phenotype $PhoP^c$) increase the expression of *pag* and repress the synthesis of approximately 20 proteins encoded by *phoP*-repressed genes (*prg*). $PhoP^c$ bacteria are attenutated for mouse virulence suggesting that *prg* are virulence genes.

By use of the transposon, Tn*phoA*, five unlinked *prg* loci were identified. In general, media conditions (starvation) that activate *pag* expression repress *prg* expression. One *prg* locus, *prgH*, was demonstrated to contribute to mouse virulence by both the oral and the intraperitoneal route. Both PrgH as well as $PhoP^c$ mutant *S. typhimurium* were found to be defective in induction of endocytosis by epithelial cells. Identification and mutation of such virulence genes will be useful in vaccine development.

Nucleotide sequence of the *prg H, prgI, prgJ,* and *prgK* genes

SEQ ID NO:10 represents the nucleotide sequence of a 5100-bp *Hind*III fragment that contains the hyperinvasive *hil* locus. Four ORFS encoding four *prg* genes are located within this DNA (see Fig. 9). The ATG start codon is underlined; the asteriks indicate the positions of the *prgH, prgI, prgJ,* and *prgK* stop codons. These *prg* loci are required for bacterial invasion of epithelial cells, full mouse virulence, and transepithelial neutrophil migration. A bacteria attenuated by a mutation in one or more of these loci can be used to vaccinate individuals against infection by the wild type pathogen.

Strains, materials and methods

All bacterial strains used in the characterization of *prg* genes are listed in Table 5.

Table 5

Strain	genotype or description	Relevant Reference or source
S. typhimurium		
14028s derivatives		
14028s	Wild type	ATCC
CS002	*phoP12*	This work
CS003	Δ*phoP* Δ*purB*	This work
CS012	*pagA1::Mu dJ*	This work
CS013	*pagB1::Mu dJ*	This work
CS119	*pagC1*::Tn*phoA phoN2 zxx*::6251 Tn*10*d-Cm	This work
CS015	*phoP-102* ::Tn*10* d-Cm	This work
CS019	*phoN2 zxx*::6251Tn*10*d-Cm	This work
CS022	*pho-24*	This work
CS023	*pho-24 phoN2 zxx*::6251Tn*10*d-Cm	This work
CS030	*phoN2 zxx*::6251Tn*10*d-Cm *phoP12*	This work
AD154	*phoP12 purB1744*::Tn*10*	Gift of E. Eisenstadt
CS031	*pho-24 purB1744*::Tn*10*	This work
IB001	*phoN2* zxx::6251Tn*10*d-Cm Δ*phoP* Δ*purB*	This work
IB002	CS030 with *prgA1*::Tn*phoA*	This work
IB003	IB002 with *pho-24 purB1744*::Tn*10*	This work
IB004	IB002 with *phoP12 purB1744*::Tn*10*	This work
IB005	CS019 with *prgA1*::Tn*phoA*	This work
IB006	CS015 with *prgA1*::Tn*phoA*	This work
IB007	CS030 with *prgB1* ::Tn*phoA*	This work
IB008	IB007 with *pho-24 purB1744*::Tn*10*	This work
IB009	IB007 with *phoP12 purB1744*::Tn*10*	This work
IB010	CS019 with *prgB1* ::Tn*phoA*	This work
IB011	CS015 with *prgB1* ::Tn*phoA*	This work
IB012	CS030 with *prgB2*::Tn*phoA*	This work
IB013	IB012 with *pho-24 purB1744*::Tn*10*	This work
IB014	IB012 with *phoP12 purB1744*::Tn*10*	This work
IB015	CS019 with *prgB2*::Tn*phoA*	This work
IB016	CS015 with *prgB2*::Tn*phoA*	This work
IB017	CS030 with *prgC1*::Tn*phoA*	This work
IB018	IB017 with *pho-24 purB1744*::Tn*10*	This work
IB019	IB017 with *phoP12 purB1744*::Tn*10*	This work
IB020	CS019 with *prgC1*::Tn*phoA*	This work
IB021	CS015 with *prgC1*::Tn*phoA*	This work
IB022	CS030 with *prgE1*::Tn*phoA*	This work
IB023	IB022 with *pho-24 purB1744*::Tn*10*	This work
IB024	IB022 with *phoP12 purB1744*::Tn*10*	This work
IB025	CS019 with *prgE1*::Tn*phoA*	This work
IB026	CS015 with *prgE1*::Tn*phoA*	This work
IB027	CS030 with *prgE2*::Tn*phoA*	This work
IB028	IB027 with *pho-24 purB1744*::Tn*10*	This work

IB029	IB027 with *phoP12 purB1744*::Tn*10*	This work
IB030	CS019 with *prgE2*::Tn*phoA*	This work
IB031	CS015 with *prgE2*::Tn*phoA*	This work
IB032	CS030 with *prgE3*::Tn*phoA*	This work
IB033	IB032 with *pho-24 purB1744*::Tn*10*	This work
IB034	IB032 with *phoP12 purB1744*::Tn*10*	This work
IB035	CS019 with *prgE3*::Tn*phoA*	This work
IB036	CS015 with *prgE3*::Tn*phoA*	This work
IB037	IB001 with *prgH1*::Tn*phoA*	This work
IB038	IB037 with *pho-24 purB1744*::Tn*10*	This work
IB039	IB037 with *phoP12 purB1744*::Tn*10*	This work
IB040	CS019 with *prgH1*::Tn*phoA*	This work
IB041	CS015 with *prgH1*::Tn*phoA*	This work
IB042	Tn5B50-380 in IB040	This work
IB043	pWKSH5 in IB040	This work
IB044	pWKSH5 in CS022	This work
CS032	*oxiA1049*::Mu d1-8 *supD10*	This work
CS033	*oxiC1048*::Mu d1-8 *supD10*	This work
CS034	*oxiE4*:: Mu d1 Δ*nadA100*	This work

Other *S. typhimurium* derivatives

AK3011-AK3314 of randomly spaced Tn*10*Δ16Δ17 insertions		Collection (19)
TT520	*srl-202*::Tn*10*	(41)
TT2979	*srl-211*::Tn*5*	(41)
TN3061	*zcf-845*::Tn*10 dcp-1 zhg-1635*::Tn*10*dCm	(41)
SH7782	*ompD*::Tn*5*	(41)
$_{x}^{\Delta}$4115	*invA*::*cat*	(13)
EE517	Δ*hil-517* (Tn5B50-380)	Gift of C. Lee
JF897	*oxiA1049*::Mu d1-8 *supD10*	(2)
JF896	*oxiC1048*::Mu d1-8 *supD10*	(2)
JF739	*oxiE4*::Mu d1 Δ*nadA100*	(2)

S. enteritidis

CDC5	clinical wild-type isolate	(45)
SM7	Str^{r} *smb*	(45)

E. coli

SM10(pRT291) plasmid pRT291 (Tn*phoA*) derived from pRK290 selecting for Tc^{r} and Km^{r}.		contains (49)
MM294(pPH1JI) (49)	contains Gm^{r} plasmid pPH1JI, which is incompatible with pRK290	
VV42(pWKSH5) plasmid pWKSH5, a derivative of pSC101 (51) that contains a 5.1 kb *HindIII* fragment of *hil* DNA including prgH		contains V.Bajaj and C.Lee

(19) Kukral et al., Journal of Bacteriology, 169:1787-1793, 1987

(41) Sanderson et al., Microbiological Reviews, 52:485-532, 1988

(13) Galan et al., Infection and Immunity, 59:3116-3121, 1990

(2) Aliabadi et al., Journal of Bacteriology, 165:780-786, 1986

(45) Stone et al., Journal of Bacteriology, 174:3945-3952, 1992

Bacteria were grown as follows: Luria-Bertani (LB) broth was used as rich medium. Antibiotics were used in the following concentrations in growth media or agar: ampicillin 100 μg/ml (Ap), chloramphenicol 25 μg/ml (Cm), gentamicin 30 μg/ml (Gm), kanamycin 45 μg/ml (Km), and tetracycline 25 μg/ml (Tc). The chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate (p-toluidine salt) (XP) was used to detect phosphatase activity on agar at a final concentration of 40 μg/ml. *p*-nitrophenyl phosphate (*p*-NPP) was used as a substrate for quantitative measurement of AP activity. Media was buffered to various pH ranges with 1 M sodium citrate. E media (Vogel-Bonner minimal) was prepared as described by Davis et al., 1980, Advanced Bacterial Genetics: A Manual for Genetic Engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. Nitrogen-, carbon-, and phosphate free medium ($N^-C^-P^-$) was prepared as described by Kier et. al., 1977, J. Bacteriol. 130:399, herein incorporated by reference.

This starvation medium was supplemented with 0.04% (wt/vol) glucose as the carbon source, 10 mM NH_4Cl as the nitrogen source, and 1 mM $NaH_2PO_4.H_2O$ as the phosphate source. The carbon concentration is one log less than described by Kier et al., *supra*.

AP activity of strains isogenic except for

mutations in the *phoP* locus was measured in cultures grown from a single colony inoculum under various oxygen tensions with or without shaking at 37°C. Anaerobic cultures were grown in an anaerobic chamber (Coy Laboratories Products, Inc.) with a gas mixture of 80% N_2, 10% O_2, and 10% CO_2 at 37°C. For acid regulation, aliquots of mid-logarithmic cultures were removed to measure initial pH and AP activity. 1M sodium citrate (pH >6.0) or 1M citric acid (pH 4.7) were added to equivalent amounts of culture to a final concentration of 50 mM citrate. Cultures were grown aerobically for two hours at 37°C and then pH and AP measurements were taken. AP activity was measured as described previously (Michaelis et al., 1983, J. Bacteriol. 154:366-374, herein incorporated by reference). AP units were calculated by the following formula: units = $\{OD_{420}/[\text{time (minutes)} \times \text{volume} \times OD_{600}]\} \times 1000$ as defined by Miller for β-galactosidase (Miller et al., 1972, Experiments in molecular genetics, p. 352-355. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.).

Standard bacterial genetic techniques were used to study *prg* loci. Bacteriophage P22HT*int*-mediated transduction was performed as according to methods known in the art. Tn*phoA* mutagenesis was performed using a broad host range plasmid (pRT291) to deliver Tn*phoA* (Taylor et al., 1989, J. Bacteriol. 171:1870, herein incorporated by reference). Transpositions of Tn*phoA* into *Salmonella* DNA were identified by use of the incompatibility plasmid pPH1JI (Taylor et al., *supra*). Screening for *phoP*-repressed genes was performed using CS031, the donor strain of the *pho-24* allele. CS031 was constructed by a P22 bacteriophage transductional cross between strains AD154 and CS022 which contains the *purB*::Tn*10* allele and the *pho-24* allele, respectively. The linkage of *pho-24* and *purB*::Tn*10* was 70%, similar to

the linkage of *purB* to other *phoP* alleles. Therefore, when P22 bacteriophage transductional crosses were performed between CS031 and the strains containing active gene fusions to *phoA*, strains could be screened for loss of fusion protein activity on acquisition of tetracycline resistance. Initial screening involved detection of loss of AP activity in approximately 70% of colonies that acquired tetracycline resistance, as they were presumed to contain the *pho-24* allele. In addition, controls were performed using strain AD154 that contains the same *purB*::Tn*10* allele linked to a *phoP* null allele, *phoP12*. Plasmid DNA was transformed into *S. typhimurium* strain LB5010 by the calcium chloride and heat shock procedure (Maclachlan et al., 1985, J. Bacteriol. 161:442).

Isolation of strains with Tn*phoA* insertions in *phoP*-repressed genes

Constitutive mutations in the *phoP* locus (phenotype $PhoP^c$) that result in increased expression of *pag* in an unregulated fashion also markedly attenuate *S. typhimurium* virulence and survival within macrophages. The virulence defect of $PhoP^c$ strains can be explained by their decreased expression of approximately 20 polypeptides encoded by *phoP*-repressed genes (*prg*).

A $PhoP^-PhoN^-$ strain (IB001) was constructed by a P22 transductional cross between CS019 and CS003. IB001 was then mutagenized with Tn*phoA* (so that background acid phosphatase, encoded by *phoN*, would not interfere with the measurement of fusion protein activity on alteration of the *phoP* locus) and 1800 individual blue colonies with PhoA fusion protein activity were isolated on LB agar plates containing XP. These colonies were the result of 18 separate matings with approximately 20 pools in each. These strains were tested for reduction of fusion protein activity on acquisition of the *pho-24* allele (CS031), which resulted in a $PhoP^c$ phenotype. AP assays were then performed on strains isogenic except for the *phoP* locus.

The PhoP^c phenotype was confirmed in these strains by preparation of whole cell protein extracts and SDS-PAGE analysis. All strains with a PhoP^c phenotype demonstrated the expected distinctive pattern of protein expression in PhoP^c strains, i.e. repressed protein species of specific sizes.

Eight strains were identified with gene fusions to *phoP*-repressed genes. As shown in Table 6, the synthesis of most *prg*::Tn*phoA* fusion proteins was fully repressed by the *pho-24* allele. While two loci had complete repression of fusion protein activity, others demonstrated only partial repression. The expression of *pag* in PhoP^c strains is 5-10 fold less than that observed after bacteria are phagocytosed by macrophages suggesting that the degree of repression of some *prg* loci may be greater when *pag* are maximally activated within acidified macrophage phagosomes.

Lower values for *prgB -phoA* fusions in strains with a wildtype *phoP* locus (Table 7B) compared to PhoP$^-$ strains (Table 7) may represent some degree of repression in the presence of PhoP.

TABLE 14

The effects of *pag:: phoA* gene fusions on *Salmonella* mouse virulence.

Strain	Genotype	LD_{50} [a]	MSI [b]	Reference
14028s	Wild type	< 20	6.13	25
CS015	*phoP102*::Tn*10-Cam*	$7.0x10^5$	0.40	25
CS585	*pagD1*::Tn*phoA*	$4.0x10^5$	0.01	15
CS1074	*pagJ1*::Tn*phoA*	$4.0x10^3$	0.56	This study
CS767	*pagK1*::Tn*phoA*	$9.0x10^4$	0.04	This study
CS1845	*pagM1*::Tn*phoA*	$3.0x10^4$	0.09	This study

[a] The 50% lethal dose was determined by intraperitoneal injection of ten mice per dilution using the method of Reed and Muench (31).

[b] The Macrophage Survival Index (MSI) was determined by dividing the mean *Salmonella* CFU recovered from macrophage cultures (performed in triplicate) 24 hours after the addition of gentamicin by the mean *Salmonella* CFU recovered from macrophages 1 hour after gentamicin was added.

16 Kier et al., 1979, J. Bacteriol., 138:155-61

25 Miller et al., 1989, Proc. Natl. Acad. Sci. USA, 86:5054-58

TABLE 15

Plasmids, strains and relevent properties

S. typhimurium strains	Relevent genotypes/information	MSI[a]	Source[b]
ATCC14028	Wild type	3.90	ATCC
CS019	*phoN2 zxx*::6251Tn*10*d-Cm		(31)
CS585	CS019, *pagD*::Tn*phoA*	0.002	(4)
CS586	CS585; *phoP*105::Tn*10*d-Tet		(4)
JSG205	ATCC14028, *msgA*::Mu*dJ*	0.01	This work
JSG225	JSG205, *phoP*105::Tn*10*d-Tet		This work
CS811	CS019, *envE*::Tn*phoA*		This work
CS812	CS811, *phoP*105::Tn*10*d-Tet		This work
CS100	ATCC14028, *phoP*105::Tn*10*d-Tet	0.01	derivitive of TT13208
JSG232	JSG205, *envF*::pGPP2		This work
JSG234	CS019, *envF*::pGPP2		This work
JSG235	JSG234, *phoP*105::Tn*10*d-Tet		This work
JSG244	JSG205, *phoP*105::Tn*10*d-Tet		This work
CS099	ATCC14028;*zxx*3024::Tn*10*Δ16Δ17*pol*-2(Whitfield *polA* amber)		This work
Other salmonellae			
Ty2	Vi positive		FDA
Salmonella paratyphi A	ATCC 9150		ATCC
Salmonella paratyphi C	ATCC 13428		ATCC
Salmonella enteriditis	Clinical isolate		VRI
E. coli Strains			
SM10λ*pir*	*thi*-1 *thr*-1 *leuB*6 *supE*44 *tonA*21 *lacY*1*recA*::RP4-2-Tc::Mu		
DH5α	F⁻ Ø 80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169*endA*1*recA*1*hsdR*17*deoRthi*-1*supE*44λ⁻ *gyrA*96*relA*1		
Other *Enterobacteriaceae*			
Yersinia enterocolitica	Clinical isolate		MGH bacteriology lab
Vibrio cholerae	Clinical isolate		Peruvian epidemic

Campylobacter fetus	Clinical isolate	MGH bacteriology lab
Citrobater freundii	Clinical isolate	MGH bacteriology lab
Klebsiella pneumoniae	Clinical isolate	MGH bacteriology lab
Shigella flexneri	Clinical isolate	MGH bacteriology lab
Shigella sonnei	Clinical isolate	MGH bacteriology lab
Morganella morganii	Clinical isolate	MGH bacteriology lab
Providencia stuartii	Clinical isolate	MGH bacteriology lab
Plasmids		
pWPL17	pBR322 containing a 2.8 Kb *HpaI* fragment from pWP061	This wor
pCAA9	pWPL17 containing a Tn*phoA* insertion in *envF*	This wor
pGP704	*pir*-dependent suicide vector	(34)
pGPP2	pGP704 containing the cloned *envF*::*phoA* gene fusion	This wor
pWP061	Cosmid clone containing the *pagC* region	(36)

[a] MSI (macrophage survival index) is calculated by dividing the number of surviving organisms at 2 hours post-infection by the number of cell asscociated organisms present after the 30 minute infection

[b] MGH, Massachusetts General Hospital, ATCC, American Type Culture Collection, FDA, Food an Drug Administration; VRI, Virus Research Institute

4 Belden et al., 1989, Infect. Immun., 57:1-7

31 Miller et al., 1989, Proc. Natl. Acad. Sci. USA, 86:5054-58

34 Miller et al., 1988, J. Bacteriol., 170:2575-83

36 Pulkkinen et al., 1991, J. Bacteriol., 173:86-93

TABLE 16

Alkaline phosphatase and ß-galactosidase gene fusion activity

Strain	Relevent Genotype	gene fusion activity[a]
JSG205	*msgA*:MudJ	461(B)
JSG244	*phoP*105::Tn*10*d-Tet *msgA*:MudJ	415(B)
JSG226	*envE*::Tn*phoA*	50(A)
JSG229	*phoP*105::Tn*10*d-Tet *envE*::Tn*phoA*	60(A)
JSG204	*pagD*::Tn*phoA*	76(A)
JSG225	*phoP*105::Tn*10*d-Tet *pagD*::Tn*phoA*	9(A)
JSG234	*envF*::pGPP2	16(A)
JSG235	*phoP*105::Tn*10*d-Tet *envF*::pGPP2	19(A)
JSG232	*msgA*::MudJ *envF*::pGPP2	10(A)

[a] (A) AP (alkaline phosphatase) or (B) ß-gal (ß-galactosidase)

Table 6

Allele	PhoP$^-$	PhoP^c	Fold Repression
prgA1::Tn*phoA*	29	7	4
prgB1::Tn*phoA*	137	27	5
prgB2::Tn*phoA*	77	19	4
prgC1::Tn*phoA*	14	1	14
prgE1::Tn*phoA*	21	5	4
prgE2::Tn*phoA*	34	6	6
prgE3::Tn*phoA*	25	6	4
prgH1::Tn*phoA*	92	2	46

In Table 6, a comparison of the effect of *phoP* locus mutations on Prg-PhoA fusion protein activity is made. $PhoP^-$ indicates that the strain assayed contains the phoP12 allele (CS030) and $PhoP^c$ indicates the strain assayed contains the pho-24 allele (CS031). Values were calculated from stationary phase cultures. The numbers denote representative values of experiments performed on three separate occasions and represent activity in units of AP as defined above.

Table 7A

Strain	Allele	Starvation Media	Rich Media
IB010	*prgB1*::Tn*phoA*	21	26
IB040	*prgH1*::Tn*phoA*	7	181
CS119	*pagC1*::Tn*phoA*	1263	102

Table 7B

Strain	Allele	Aerobic	Microaerophilic	Anaerobic
IB010	*prgB1*::Tn*phoA*	33	777	1521
IB040	*prgH1*::Tn*phoA*	142	85	41
CS119	*pagC1*::Tn*phoA*	431	173	81

Table 7C

Strain	Allele	pH 4.5	pH 7.0
IB010	*prgB1*::Tn*phoA*	332	26
IB040	*prgH1*::Tn*phoA*	8	18
CS119	*pagC1*::Tn*phoA*	145	27

Table 7 demonstrates the effects of environmental conditions on the *in vitro* regulation of *prg* loci. Table 7A shows the effect of starvation on *prg* and *pag* expression. Starvation medium ($N^-C^-P^-$) (17) contained 0.04% glucose, 10 mM NH_4Cl, and 1 mM $NaH_2PO_4.H_2O$. The fusion protein activity for starvation media was measured after 48 hours of growth (OD_{600} = 0.5) while that in rich media (LB) was measured in late-logarithmic growth (OD_{600} = 1.0).* All cultures were grown aerobically.

Table 7B shows the effect of oxygen tension on expression of *phoP*-activated and *phoP*-repressed genes. Expression in rich medium is compared under aerobic conditions at stationary phase (OD_{600} > 1.4), microaerophilic (OD_{600} = 0.8), and strict anaerobic conditions with 80% N_2, 10% O_2, and 10% CO_2 (OD_{600} = 0.6) after 24 hours of growth.*

Table 7C shows the effect of pH on the expression of fusion protein activity of *prg* and *pag* loci. Expression was measured from cultures grown to logarithmic growth (OD_{600} = 0.5) in LB media buffered to various pHs with sodium citrate. All the numbers represent activity in units of AP as defined above.

<u>Chromosomal location of *prg*::Tn*phoA* loci</u>

prg::Tn*phoA* linkage analysis was performed to a bank of strains with randomly spaced Tn*10*Δ16Δ17 insertions to determine chromosomal locations and whether *prg*::Tn*phoA* alleles were unlinked loci. The *prg*::Tn*phoA* insertions were in five distinct linkage groups. Three alleles, *prgE1-3*::Tn*phoA* were identically linked to the Tn*10*Δ16Δ17 insertion of AK3091(26%) and two other alleles, *prgB1-2*::Tn*phoA* were similarly linked to the Tn*10*Δ16Δ17 insertion of AK3190 (94%), AK3249 (89%), and AK3186 (50%). Another allele, *prgH1*::Tn*phoA*, was found to be 37% linked to the Tn*10*Δ16Δ17 insertion of strain AK3304. The other two *prg* alleles did not demonstrate linkage to the bank of strains tested. The chromosomal DNA of these two strains was

analyzed by Southern hybridization analysis using a portion of Tn*phoA* as a probe, and a rough physical map of the sites located adjacent to the Tn*phoA* insertion was determined. These alleles, *prgA* and *prgC*, had different restriction endonuclease sites surrounding the Tn*phoA* insertions. In addition, the repression of *prgA* and *prgC* fusion protein activity in strains with the *pho-24* mutation was different; *prgC* was completely repressed, while *prgA* was only partially repressed indicating that these loci are different. Thus, five unlinked loci encoding envelope proteins repressed in the $PhoP^c$ phenotype were identified.

Though three *prg* loci were identified that were linked to transposon insertions, none of the Tn*10*Δ16Δ17 insertions had a known map location. The physical map location of two of these transposon insertions, AK3249 and AK3304, was analyzed using *Xba*I restriction endonuclease digestion and pulse field gel electrophoresis (PFGE). Since Tn*10*Δ16Δ17 contains a single *Xba*I site, these Tn*10*Δ16Δ17 insertions can be assigned to a specific *Xba*I fragment of known map location (Liu et al., 1992, J. Bacteriol. 174:16622). AK3249 was assigned to 28-32 min, while AK3304 was assigned to either end of the 58-70 minute fragment. Further P22 transduction to known markers in those regions was performed. The Tn*10*Δ16Δ17 insertion of strain AK3249 and *prgB1*::Tn*phoA* were found not to be linked to the Tn*10* insertion of strain TN3061 (6% linked to *dcp*), which has a transposon insertion at 28 min, or to the *ompD*::Tn*5* insertion of strain SH7782 at 32 min. *prgH1*::Tn*phoA* was found to be very weakly linked to the *srl202*::Tn*10* insertion of strain TT520 (<0.1%) at 59 minutes. These data indicate that *prg* are unlinked on the *Salmonella* chromosome, consistent with the function of PhoP/PhoQ as global regulators.

The chromosomal location of Tn*phoA* insertions in *phoP*-repressed genes (*prg*::Tn*phoA*) was determined by linkage analysis to a bank of strains with Tn*10*Δ16Δ17 insertions (Kukral et al., 1987, J. Bacteriol. 169:1787, herein incorporated by reference). Cells with Tn*phoA* insertions were spread on LB agar plates containing 10 μg/ml tetracycline and 40 μg/ml XP. Then P22 lysates grown on strains with Tn*10*Δ16Δ17 insertions were spotted onto plates with a multiprong inoculator. After overnight inoculation, plates were reviewed for linkage by looking for mixed blue and white colonies. Linkage was confirmed and quantitated by carrying out individual transductional crosses between the Tn*10*Δ16Δ17 containing strains and the strain with the Tn*phoA* insertion. After selection for the Tn*10*Δ16Δ17 encoded tetracycline resistance, strains were scored for loss of blue color and Tn*phoA* encoded kanamycin resistance. Some Tn*phoA* strains were found to be linked to Tn*10*Δ16Δ17 strains with no known map location. Two of these Tn*10*Δ16Δ17 insertions were physically mapped using PFGE following *Xba*I restriction endonuclease digestion. Based on physical mapping, linkage analysis to other transposon insertions by P22 bacteriophage transduction was determined as necessary.

Chromosomal DNA was prepared as described by Mekalanos, 1983, Cell 35:253, herein incorporated by reference, using Proteinase K instead of Pronase. Purification of plasmid DNA was performed by standard methods. Restriction endonuclease digestion was performed according to the recommendations of the manufacturer (New England Biolabs). DNA, size fractionated in agarose gels, was transferred to Genescreen Plus membranes (New England Nuclear/Dupont, Boston, MA) for blot hybridization by the method of Southern well known in the art. DNA probes were purified from agarose gels by the freeze-squeeze method (Tautz et al., 1983, Anal. Biochem. 132:14) and radiolabelled with [^{32}P]dCTP by the random primer method

(Feinberg et al., 1983, Anal. Biochem. 132:6).

Cloning genes from Tn*pho A* fusions

The gene encoding *prgH* has been cloned using methods described below. The plasmid, pIB01, containing the *prgH* gene has been deposited with the American Type Culture Collection on July 9, 1993 (Rockville, MD) and has received ATCC designation ATCC 75496. Fig. 5 shows the partial DNA sequence of *prgH* (SEQ ID NO: 3). Fig. 9 shows the location and sequence of the entire *prgH* gene.

The genes described herein which have been identified by Th*phoA* insertion can be cloned using methods known in the art (Beattie et al., 1990, J. Bacteriol. 172:6997). Chromosomal For example, DNA of each strain containing a *prg*::Tn*phoA* gene fusion is digested with a restriction enzyme such as BamH1 which cuts at a single site in Tn*phoA* maintaining the fusion junction, *phoA* sequences and the *neo* gene. Similarly, a plasmid such as pUC19 is digested with the same enzyme. Digested chromosomal and plasmid DNA are ligated overnight at 15°C and transformed into competent *E. coli*. Transformations are plated on LB agar containing ampicillin and kanamycin to select for the *bla* gene of pUC19 and the *neo* gene of TnphoA. The chromosomal DNA containing the *prg*::Tn*phoA* gene fusion can then be sequenced using standard methodology described above, such as the SEQUENASE® (United States Biochemical) kit. Universal primer (United States Biochemical) corresponding to DNA sequences in the plasmid or Tn*phoA* primer (5'-AATATCGCCCTGAGCA-3') (SEQ ID NO:4) corresponding to bases 71 to 86 of Tn*phoA* can be used as primers.

To clone the wild type gene, a fragment of chromosomal DNA flanking Tn*phoA* sequences can be used to screen a cosmid gene bank of wild type *Salmonella* strain ATCC 10428 using methods described above for cloning wild type *pagC*.

Environmental regulation of prg loci

Since PhoP/PhoQ are environmentally responsive regulators, the effects of different growth conditions on *prg::TnphoA* expression were tested. The growth rate of *prg::TnphoA* insertions was comparable to wild-type organisms under all conditions. The expression of all *prg* loci was maximal in late logarithmic growth phase when bacteria were grown in rich (LB) media. An example of this is the comparison of values of *prgH::TnphoA* expression in Table 7A (rich media and stationary growth) and Table 7C (pH 7.0, log phase). Since the expression of *pag* loci was maximal in starvation (which only reaches a maximal OD_{600} = 0.5) and stationary growth phase, this was consistent with a reciprocal relationship between the expression of *pag* and *prg* loci. Further analysis of *prg* loci expression under starvation conditions confirmed this reciprocal relationship (Table 7A). *prgH* expression was repressed (Table 7A) and maximal in starvation conditions, in contrast to the induction of *pag* expression (Table 7A). *prgs*. Further analysis of *prg* loci expression under other *prg* were minimally affected under starvation conditions, in contrast to the induction of *pag* expression when bacteria were starved (Table 7A).

Because of its role in bacterial-mediated endocytosis (BME), the effect of oxygen tension in rich medium on *pag* and *prg* expression was also tested (Table 7B). Different but not reciprocal regulation of *pag* and *prg* loci was found on growth at different oxygen tensions. Though *pagA* and *pagB* loci were minimally affected by growth at different oxygen tensions, the *pagC* virulence locus was approximately 5 fold repressed when bacteria were grown anaerobically as compared to aerobic growth (Table 7B). Variability was also noted in the expression of *prg* loci in response to growth conditions in the absence of oxygen. One loci, *prgH*, was repressed three-fold in anaerobic growth, while another locus, *prgB*, was induced almost 50-fold when grown anaerobically (Table 7B). Other *prg* loci had minimal change in fusion protein expression as a result of different

oxygen tensions in the growth media.

Low pH conditions also had a variable effect on *prg* expression (Table 7C). The expression of *pagC* fusion protein activity was induced under acid conditions as previously known. When bacteria were grown to mid-logarithmic growth, no significant induction of the relative repression of *prgH* expression was noted in media of low pH, while *prgB* expression was induced on exposure of bacteria to low pH (Table 7C). Hence, loci maximally expressed under diverse environmental conditions can all be repressed by the $PhoP^c$ phenotype.

Acid sensitivity was tested by the method of Foster et. al., 1990, J. Bacteriol. 172:771, herein incorporated by reference. Strains were grown aerobically in E media and 0.4% glucose at 37°C to an OD_{600} of 0.5. The pH of the bacterial culture was decreased to near 3.3 by the addition of 1 M hydrochloric acid. An aliquot was taken immediately (t_o), the remainder of the culture was incubated further at 37°C with subsequent aliquots removed at 40 min (t_{40}) and 80 min (t_{80}) time points. The pH of the cultures remained near 3.3. The aliquots were diluted 1:10 in cold PBS, washed and resuspended in normal saline prior to plating serial dilutions for colony forming units.

prgH is a virulence locus for *S. typhimurium*

Since the $PhoP^c$ phenotype resulted in virulence attenuation and repressed the synthesis of approximately 20 proteins, the virulence of strains with single mutations in *prg* loci was tested (Table 8). Strains with *prg*::Tn*phoA* insertions were screened for virulence defects by intraperitoneal injection of approximately 150 organisms into BALB/c mice. Controls were also performed with wild-type bacteria. A significantly longer time course of clinical disease progression was observed with a *prg* mutant strain compared to wild type bacteria. Mice injected intraperitoneally with strains containing the *prgH1*::Tn*phoA*

insertion developed clinical signs of typhoid fever, such as a "scruffy "phenotype (fever and piloerection) and hepatosplenomegaly in approximately 10-14 days, compared to approximately 24 hours for the wild type bacteria. Despite the extended time course of disease development, all the mice eventually died. Disease progression of mice injected with other strains containing *prg*::Tn*phoA* insertions showed a similar pattern of illness to that of wild type bacteria.

Table 8

			LD_{50}
Intraperitoneal injection			
	14028s	Wild type	<10
	IB040	*prgH1*	5.6×10^1
	CS015	*phoP-102*	6.7×10^5
	IB041	*prgH phoP-102*	1.2×10^7
Oral inoculation			
	14028s	Wild type	6.5×10^4
	IB040	*prgH1*	6.5×10^5

Table 8 shows the effect of the *prgH1*::Tn*phoA* mutation on *Salmonella* mouse virulence. Strains were isogenic and administered by intraperitoneal injection and oral inoculation in 35 day old BALB/c mice. The number of animals used at bacterial dilutions near the LD_{50} for each allele is listed in parentheses. The LD_{50} determinations were repeated on three separate occasions.

Further testing of the LD_{50} of strains containing *prgH* mutations was performed. *prgH* mutants were determined to have an LD_{50} of approximately 60 organisms compared to a value of <10 for wild type bacteria. Due to the difficulty in accurately delivering organisms in small doses to mice, a strain with a mutation in both *prgH* and *phoP* was constructed. The PrgH$^-$PhoP$^-$ strain had greater than a 10 fold increase in LD_{50} compared to CS015, an isogenic PhoP$^-$ strain (Table 8). The combined effect of the two mutations further documented that the *prgH1*::Tn*phoA* mutation attenuated *S. typhimurium* virulence and indicated that mutations which affected two phases of PhoP/PhoQ regulated gene expression were additive in their effect on virulence. Strains with *prgH1*::Tn*phoA* insertions were also tested for virulence when administered by the oral route. A 10 fold decrease in virulence (increase in LD_{50}) was observed (Table 8).

Further analysis of the efficiency of strains with *prgH1*::Tn*phoA* insertions in crossing the mucosal barrier was tested by competition experiments with wild-type bacteria. During the first 72 hours after oral inoculation with mutant bacteria, no *prgH1*::Tn*phoA* mutants were recovered from the bloodstream of mice compared to control experiments in which organisms were routinely isolated from the blood of mice inoculated with wild type bacteria. Other strains with *prg* mutations were also tested for virulence defects by the oral route, but no significant change in virulence was observed.

Mouse virulence studies were carried out as follows. Bacteria were grown aerobically at 37°C to stationary phase, washed with LB, and diluted in normal saline. 35 days old (16-18g) female BALB/c mice were purchased from the Charles River Breeding Laboratories, Inc. (Wilmington, MA). Diluted bacterial samples in saline were injected intraperitoneally with an inoculum of 0.1-0.15 ml. Bacteria were administered orally as a 0.5 ml bolus to mice fasted for 2 hours, via a 2 inch straight, 18 gauge stainless steel animal oral feeding needle (Harvard Apparatus, Inc., South Natick, MA) under mild 2-bromo-2-chloro-1,1,1-trifluoroethane (Halothane) anesthesia. The number of organisms administered was quantitated by plating for cfu/ml on LB agar. Mouse 50% lethal dose (LD_{50}) values were determined by standard methods (Reed and Muench, 1938, Amer. J. Hygiene 27:493). The LD_{50} determinations were repeated on three separate occasions. Competition assays were performed after bacteria were administered orally to mice as above. Bacteremia was assessed on days 1-4 from tail bleeds or intracardiac punctures with 50 µl of blood plated immediately and after growth in LB broth at 37°C overnight. Spleen and intestinal harvests were performed on days 1-6 with organs homogenized in 3 mls of 0.9% sodium chloride. Samples and cultures were plated in serial dilutions. *S. typhimurium* was confirmed by characteristic growth (black colonies) on Hektoen-enteric agar (Difco Laboratories) and by the macroscopic slide agglutination test with *Salmonella* rabbit serum Group B (Antigens 4, 5, 12) (Fisher Scientific).

Mutations in oxygen-induced genes do not affect mouse virulence

Both *prgH* and *pagC* loci were shown to be repressed by anaerobic growth and required for full virulence, thus suggesting that a shift from anaerobic to aerobic conditions might serve as a general signal for induction of virulence genes. Strains with mutations in oxygen-inducible loci

(Aliabadi et al., 1986, J. Bacteriol. 165:780) were constructed. ATCC14028s derivatives with *oxiA*, *oxiC*, and *oxiE* mutations were made (termed CS032, CS033, CS034, respectively). These strains were as virulent as wild type bacteria. Though these gene fusions could still mark operons containing virulence genes, this data suggests that these loci are not essential to full virulence and that oxygen induction is not always correlated with virulence function.

prgH mutants have normal survival within macrophages

Since the PhoP^c phenotype resulted in a defect in bacterial survival within macrophages, the effect of this mutation on the synthesis of a *prgH*-encoded protein was tested. A strain with the *prgH1*::Tn*phoA* insertion was tested for intracellular survival within bone marrow-derived macrophages from BALB/c mice and J774.2 cells, a macrophage derived cell line. No defect in intracellular survival was observed. A strain with a *prgB1*::Tn*phoA* insertion was also tested and found not to have a defect in survival within macrophages.

Assays to determine bacterial survival within macrophages were performed as described by Buchmeier al., 1989, Infect. Immun. 57:1, herein incorporated by reference. Bacteria grown to stationary-phase were opsonized for 30 minutes in normal mouse serum before exposure to cultured bone marrow-derived macrophages harvested from BALB/c mice. One hour after infection, gentamicin 10 μg/ml was added to kill extracellular bacteria. All time points (1, 4, and 24 hr) were done in triplicate and repeated on three separate occasions.

Cultured bone marrow macrophages were harvested from BALB/c mice purchased from the Charles River Breeding Laboratories. J774.2 macrophages were cultured in Dulbecco's minimal essential medium with 10% fetal bovine serum (DMEM/10%FBS).

prg::Tn*phoA* insertions do not suppress the phenotypes of PhoP mutants

Several phenotypes of *phoP* mutants, including defensin and acid sensitivity as well as mouse virulence attenuation, were tested for suppression on addition of a *prg*::Tn*phoA* mutation. To test the ability of a *phoP* mutation to suppress the synthesis of *prg* products, PhoP mutant strains isogenic except for *prg*::Tn*phoA* mutations were constructed and tested for mouse virulence, where suppression would involve an increase in virulence, or decreased acid and defensin sensitivity. *prg*::Tn*phoA* insertions had no effect on the virulence phenotypes of $PhoP^-$ bacteria. These results indicate that the *prg*::Tn*phoA* mutations tested did not suppress the PhoP null phenotype as single mutations.

PrgH and $PhoP^c$ mutants are defective in bacterial-mediated endocytosis by cultured epithelial cells

The BME of *prg*::Tn*phoA* and $PhoP^c$ *S. typhimurium* strains was tested. The following observations (described herein) suggested that *prg* genes may be involved in bacterial-mediated uptake by eucaryotic cells: *prgH1*::Tn*phoA* was shown to be located at 59′ on the bacterial chromosome, a location where other genes essential to invasion are clustered; *prgH* mutants were shown to be defective in competition with wild type organisms on reaching the bloodstream of mice in the first 72 hours after oral ingestion; and the expression of one *prg* locus, *prgB*, was dramatically induced under anaerobic growth conditions. Strains with *prgH* and *pho-24* mutations had a significant reduction (p-value < 0.01) in their ability to induce uptake by Madin-Darby canine kidney (MDCK) polarized epithelial cells compared to wild-type bacteria. Other *prg* strains with Tn*phoA* insertions did not demonstrate a statistically significant defect in BME by epithelial cells (Table 9). The adherence of strains defective in BME was unaffected by the *prgH*::Tn*phoA* insertion when determined by cell-

associated cfu/ml before the administration of gentamicin (Table 9) and by microscopy.

To assay bacterial adherence and uptake of bacteria by epithelial cells, bacterial strains were grown at 37°C without shaking (microaerophilic) to a final density of approximately $2x10^8$ colony forming units (cfu)/ml. Assays were performed by seeding 10^5 MDCK cells/well in 24-multiwell tissue culture plates. Cells were incubated overnight at 37°C in 5% CO_2/95% air atmosphere in DMEM/10%FBS without antibiotics until >80% confluent. The adherence and invasion assays were carried out according to the protocol of Lee and Falkow, 1990, Proc. Natl. Acad. Sci. USA 87:4304, herein incorporated by reference.

Table 9

Strain	Genotype	Adherence	Invasion
14028s	Wild type	4.2%	3.8%
SM7	Str^r *smb*	---	0.6%*
CS119	*pagC1*::Tn*phoA*	---	1.9%
IB005	*prgA1*::Tn*phoA*	---	7.6%
IB010	*prgB1*::Tn*phoA*	---	2.9%
IB020	*prgC1*::Tn*phoA*	---	1.5%
IB025	*prgE1*::Tn*phoA*	---	1.9%
IB040	*prgH1*::Tn*phoA*	5.7%	0.1%*
CS022	*pho-24*	1.9%	0.06%*
IB043	pWKSH5 in IB040	---	17.5%*
IB044	pWKSH5 in CS022	---	0.09%*

In Table 9, the effect of *prg*::Tn*phoA* insertions on *Salmonella*-mediated endocytosis by MDCK epithelial cells is shown. Microaerophilically grown bacterial strains were assessed for changes in adherence and invasion. Adherence was determined as the percentage of bacteria adhered to the cells after centrifugation and 30 minute 4°C incubation/ total number of bacteria added to each well. Invasion was determined as the percentage of bacteria that had invaded after a two hour incubation with gentamicin/ total number of bacteria added to each well. There was no difference between *S. typhimurium* wildtype and *S. enteritidis* CDC5 wildtype strains with respect to adherence and invasion frequency. The asterisk (*) represents statistical significance by variance analysis of the invasion data done in triplicate compared to wild-type (p-value < 0.01).

The confluent MDCK monolayers were washed three times with PBS, then 0.9 ml of cold DMEM/10%FBS was added to each well. Bacteria were washed in LB and resuspended in an equivalent volume of DMEM/10%FBS. Approximately $5x10^7$ bacteria were added/well. The plates were spun at 500 rpm at 4°C for 10 minutes, then incubated at 4°C for 30 minutes. Adherent bacteria were recovered by washing the plates three times with phosphate-buffered saline (PBS), lysing the epithelial cells in 0.5 ml of 1% Triton-X-100/PBS, and plating for cfu/ml on LB agar. A morphologic assessment of adherence was also performed by staining bacterially infected epithelial cell monolayers grown overnight on coverslips for 7 minutes in 1 μg/ml 4′ 6-diamidino-2-phenylindole (DAPI). These DAPI stained coverslips were examined by both fluorescent and phase contrast microscopy using a Leitz Laborlux 12 microscope.

Invasion or bacterial-mediated endocytosis (BME) was assessed by allowing bacteria to adhere as described above. Plates containing bacteria and epithelial cells

were incubated for two hours at 37°C in a 5% CO_2/95% air atmosphere. Each well was washed three times with PBS to remove bacteria not associated with cells. DMEM/10%FBS supplemented with 10 μg/ml gentamicin was then added to kill extracellular bacteria. After 90 minutes of incubation, the cell monolayers were washed three times with PBS and the viable intracellular bacteria were released by vigorously pipetting with 0.5 ml of 1% Triton X-100/PBS. An invasion deficient *Salmonella enteritidis* mutant and an invasive clinical wild-type isolate of *S. enteritidis* were used as controls for BME. Viable bacteria were quantitated by plating for cfu/ml on LB agar medium. All assays were done in triplicate and repeated at least three times.

MDCK epithelial cells were used between passage 40-58 to maximize bacterial adherence and invasion. Epithelial cell lines were cultured in DMEM/10% FBS and 1% penicillin/streptomycin solution at 37°C in a 5% CO_2 atmosphere.

To assay bacterial defensin sensitivity, NP-1 defensin was purified from rabbit peritoneal neutrophils according to methods known in the art (Selsted et al., 1985, J. Biol. Chem. 260:4579; Selsted et al., 1984, Infect. Immun. 45:655). Typically, 10^5 bacteria in 0.5% tryptone in 100 μl volume were exposed to 50-100 μg of defensin/ml at 37°C for 2 hours. The reactions were stopped by diluting the reaction in 0.9% NaCl. Appropriate dilutions were plated to determine the cfu/ml of surviving bacteria. Assays were performed in duplicate at least twice for each strain. Appropriate assays with sensitive ($PhoP^-$) and resistant (wild-type) strains were performed as controls.

Mapping of *prgH*

The location of *prgH* relative to other invasion loci at 59 minutes was determined using linkage analysis. P22 transduction linkage analysis indicated that the Tn*10*Δ16Δ17 of strain AK3304 had similar linkage to *invA* (40%) and *prgH* (37%); however, *invA* was not linked to sorbital. The *prgH1*::Tn*phoA* insertion was found to be linked (99.6%) to the transposon insertion of EE517, a strain with a 8.5 kilobase deletion adjacent to the Tn5B50- 378 insertion of *hil*.

A physical map of the restriction endonuclease sites surrounding the Tn*phoA* insertion of strain IB037 was made (Fig. 4) revealing no similarities to the known restriction endonuclease map of the *invA-E* region. Plasmids containing the cloned *inv* and *hil* DNA were then used as probes in Southern hybridization analysis of chromosomal DNA from wild type ATCC10428s and IB040 bacteria containing the *prgH1*::Tn*phoA* insertion. When a plasmid which contains other invasion loci highly linked to *invA-E* (*invH*, *invF*, and part of *invG*) was used as a probe, no differences in hybridization pattern was found between wild type bacteria and strain IB040 indicating that *prgH* was not located within the *inv* region. However, when a plasmid containing a 5 kb region immediately downstream of the Tn5B50-380 insertion of *hil* was used as a probe, the *prgH1*::Tn*phoA* insertion was demonstrated to be located within this region. By use of the known restriction map of the *hil* locus (Lee et al., 1992, Proc. Natl. Acad. Sci. USA 89:1847) and the known restriction endonuclease sites of Tn*phoA*, the physical map of this area and the relationship of *prgH1*::Tn*phoA* within it were further defined (Fig. 4). The *prgH1*::Tn*phoA* insertion was oriented so that the direction of transcription of the *phoA* fusion protein was opposite to that of the Tn5B50 insertions that confer the *hil* phenotype and contain a constitutive neomycin

promoter that is transcribed out of the transposon (Fig. 4). Although *prgH* was found to be located within the *hil* locus, this gene is unique in that it is oppositely transcribed and unlike any other genes identified within the *hil* locus, *prgH* is regulated by the *phoP* regulon.

Since it was possible that a protein whose expression was altered by the Tn*5*B50-380 insertion might alter the expression of *prgH*, strains containing both insertions were constructed and the *prgH-phoA* fusion protein activity compared under different environmental conditions. When bacteria were starved or grown anaerobically, derepression of fusion protein activity was observed. Table 11 shows the effect of the Tn*5*B50-380 insertion on expression of *prgH* fusion protein activity.

Table 11

Strain	Allele	Starvation	LB (aerobic)	LB(anaerobic)
IB040	*prgH1*::Tn*phoA*	5	142	41
IB042	Tn*5*B50-380 *prgH1*::Tn*phoA*	46	248	227

This data demonstrates that the Tn5B50-380 insertion increased *prgH* expression, even though *prgH* transcription was opposite to that of the Tn*5*B50-380 encoded neomycin promoter. Starvation (repressing conditions for *prg*) indicates that bacteria were grown aerobically for 48 hours in starvation medium ($N^-C^-P^-$) containing 0.04% glucose, 10 mM NH_4Cl, and 1 mM $NaH_2PO_4.H_2O$. LB (aerobic) indicates that bacteria were grown in Luria-Bertani broth (rich media) to late logarithmic growth (nonrepressing conditions) (OD_{600} >1.0). LB (anaerobic) indicates that bacteria were grown under strict anaerobic conditions for 24 hours (OD_{600} = 0.6). All the numbers represent activity in units of AP as described above.

To rule out the possibility that the BME defect of the *prgH* mutant was an artifact of the PhoA fusion protein produced, complementation analysis was performed with a plasmid (pWKSH5) containing a 5.1 kb *HindIII* fragment which included the *hil* and *prgH* loci. The plasmid was crossed into PrgH (IB040) and PhoP^c (CS022) mutant bacteria to create strains IB043 and IB044, respectively. The BME phenotype of the PrgH mutant was similar to wild-type with the same plasmid insertion. The BME phenotype of the PhoP^c mutant was not complemented by this plasmid. These results indicate that a gene product altered in synthesis as a result of the *prgH*::Tn*phoA* insertion was necessary for BME.

Using a strain with a *phoP/phoQ* locus mutation that constitutively simulates the environmental activation of *pag* (phenotype PhoP^c), five unique *phoP*-repressed loci encoding envelope proteins were defined. *phoP*-repressed genes (*prg*) were found to be widely spaced on the chromosome and the expression of *prg* loci was repressed under starvation conditions, when *pag* loci were induced (Table 10).

Table 10

Environment	*pag*	*prg*
media	starvation	rich
O_2	aerobic - *pagC* anaerobic - *prgB*	aerobic -*prgH*
pH	3.3-5.5	3.3-5.5 -*prgB* >6.0 - *prgH*
mammalian cell	macrophage	epithelial

PrgH was shown to lie between two Tn5B50 insertions that confer the *hil* phenotype. Since deletion mutants in this region have been demonstrated to also have defects of BME, and the BME defect of *prgH* mutants can be complemented with a plasmid containing this locus, it is

possible that a protein not synthesized as a result of the *prgH1*::Tn*phoA* insertion promotes BME (Fig. 4).

Contrary to the expectation that genes essential to the *hil* phenotype would be induced under microaerophilic conditions similar to what was found for *prgB*, *prgH* expression was maximal during aerobic growth and the Tn*5*B50-380 insertion, which results in a *hil* phenotype, derepressed expression of *prgH*. In addition, the direction of transcription predicted by the *prgH1*::Tn*phoA* insertion is opposite to that of the Tn*5*B50-380 encoded neomycin promoter associated with the *hil* phenotype suggesting that a regulatory protein interrupted by or transcribed from the Tn*5*B50-380 insertion affects the expression of *prgH*.

In view of the observation that pWKSH5, a plasmid containing *prgH* (*hil*), did not complement PhoP^c bacteria for BME, it is possible that other invasion genes may also be regulated by PhoP/PhoQ. If *prgH* was expressed from pWKSH5, despite the presence of the *pho-24* mutation, this suggest that other genes repressed as part of the PhoP^c phenotype are necessary for BME.

The identification and characterization of *prgH* has shown that PhoP/PhoQ oppositely regulate factors necessary for bacteria to enter or to survive within mammalian cells, further documenting the importance of gene regulation to bacterial virulence. The identification of *prg* loci can be used to study the regulation of bacterial genes after infection of mammalian cells. Understanding the regulation of virulence genes, such as *prgH* can also be used to attenuated pathogenic bacteria for the development of new live vaccines for typhoid fever.

Role of *prg* genes in virulence

The *prg* locus, *prgH*, was found to contribute to mouse virulence when *S. typhimurium* was administered by

both the oral and intraperitoneal routes. PrgH as well as PhoP^c mutants were further found to be defective in bacterial-mediated uptake by epithelial cells suggesting that an inability to cross epithelial barriers might contribute to the attenuation of virulence observed. Competition studies in mice after oral ingestion of bacteria further supported that *prgH* mutants were defective in transcytosis across the intestinal epithelial barrier. Therefore, at least two phases of PhoP/PhoQ regulated protein expression essential to bacterial virulence have been defined. In one phase, *prg* expression promotes bacterial mediated endocytosis by epithelial cells (Table 10), while in another phase, *pag* expression promotes survival within macrophages.

Systemic pathogens, such as *Salmonella*, may encounter more complex and varied environments than may be encountered by mucosal pathogens. The achievement of intermediate states of *pag* and *prg* expression could be essential to virulence at some stage of the infectious cycle. Consistent with this concept was the lack of uniformity observed in the expression of *pag* and *prg* on growth at different oxygen tensions and pH conditions. These data may also indicate that not all regulation of *pag* and *prg* is mediated directly through PhoP and PhoQ. Given the function of PhoP as a transcriptional regulator, it is likely that *prg* loci repression occurs at the level of transcription.

The approach of defining genes repressed by the *pho-24* mutation has led to the discovery of at least one virulence locus, *prgH*, which can be mutated to attentuate the bacteria for vaccine purposes.

Attenuation of Bacterial Virulence by Constitutive Expression of Two-component Regulatory Systems

The virulence of a bacterium can be attenuated by inducing a mutation which results in the constitutive expression of genes under the control of a two-component

regulatory system or by inducing a mutation that inactivates a gene under the control of the two-component systems. A balance between the expression of the genes under the control of the two-component system, e.g., between *pag* and *prg* gene expression, and possibly between two-component system regulated genes and other genes, is necessary for full virulence. Mutations that disrupt this balance, e.g., mutations that cause the constitutive expression of a gene under the control of the two-component system, or a mutation that inactivates a gene under the control of the two-component system, e.g., the *pag* gene, reduce virulence.

Constitutive mutations in two-component regulators can be identified by the use of a strain containing a recorder gene fusion to a gene regulated by the two-component system. Such gene fusions would most typically include DNA encoding the *lacZ* gene or AP fused to a gene under the control of the two-component system. Strains containing fusions that are (as compared to wild type or parental strains) highly expressed in an unregulated fashion, i.e., constitutive, can be detected by increased color on chromogenic substrates for the enzymes. To detect constitutive mutations a cloned virulence regulator could be mutagenized e.g., by passage through an *E. coli* strain defective in DNA repair or by chemical mutagenesis. The mutated DNA for the regulator would then be transferred to the strain containing the gene fusion and constitutive mutations identified by the high gene fusion expression (blue color in the case of a *lacZ* fusion grown on media containing X-gal). Constitutive mutations in a component of a two-component regulatory system could also be made by *in vitro* mutagenesis after other constitutive mutations have been sequenced and a specific amino acid change responsible for the constitutive phenotype identified. Putting several amino

acid changes that all result in a PhoP constitutive phenotype would result in a decreased frequency of reversion by spontaneous base changes. A constitutive mutation could also be constructed by deletion of the portion of the amino terminus of the phospho-accepting regulator which contains the phosphoacceptor domain e.g., deletion of sequences encoding amino acids amino terminal to amino acid 119 in the *phoP* gene or deletion of analogous phospho accepting sequences in genes of other two-component regulatory systems. This could result in a conformational change similar to that induced by phosphorylation and result in increased DNA binding and transcriptional activation.

Attenuation of virulence: deletion in the phoP/phoQ regulon

As discussed above, the PhoP regulon is essential to full virulence of *Salmonella*. This regulon is composed of two genes, PhoP and PhoQ located in an operon, and over 40 genes they positively and negatively regulate (pag and prg, respectively).

PhoP null *S. typhimurium* mutants have been demonstrated to be markedly attenuated and also effective vaccine strains when studied in the BALB/c mouse model of typhoid fever. This phenotype is likely the result of multiple, phoP-activated virulence genes, as transposon insertions in multiple different phoP-activated genes have been independently demonstrated to decrease *S. typhimurium* virulence. *S. typhimurium* mutants deleted for genes essential to aromatic amino acids (aroA null or aroC/aroD null mutants) are also markedly attenuated in the mouse model. However, testing of *aroC/aroD* mutants in humans has shown that although these strains are immunogenic, bacteremias and side effects such as fever have been noted at doses as low as 10^5 to 10^7 organisms administered as a single oral dose (J. Clin. Invest. 90:412-420).

It has now been found that a large deletion in a global regulator of *Salmonella* virulence, i.e., the PhoP/PhoQ operon, significantly decreases the virulence of the bacteria. This mutation, the result of a 1 kB deletion of DNA within the *phoP/phoQ* locus, was initially made in *S. typhimurium* and subsequently transferred via homologous recombination to *S. typhi*. In order to confer an even greater margin of safety in construction of this vaccine, it was created in a strain background deleted for genes essential to aromatic amino acids and carrying the histidine G46 mutation, a mutation rendering the organism auxotrophic for histidine. The resulting strain, *S. typhi* TyLH445, offers several advantages over existing vaccine candidates, most notably, immunogenicity without transient bactermia.

Use

The *Salmonella* cells of the invention are useful as sources of immunological protection against diseases, e.g., typhoid fever and related diseases, in an animal, e.g., a mammal, e.g., a human, in particular as the basis of a live-cell vaccine capable of colonizing the inoculated animal's intestine and provoking a strong immune reaction. Appropriate dosages and conditions of administration of such a live, attenuated vaccine are known in the art, e.g., as described in Holem et al., Acute Enteric Infections in Children, New Prospects for Treatment and Prevention (1981) Elsevier/North-Holland biomedical Press, Ch. 26, pp. 443 et seq. (Levine et al.), hereby incorporated by reference, and are described in the examples below.

Advantages

One advantage of the invention is that the bacterial cells are attenuated as a result of a mutation(s), i.e., the *phoP/phoQ* operon, that directly affect a virulence pathway. Another advantage is that

the bacterial cells have mutations in two completely different attenuating genes, i.e., the aromatic amino acid synthesis pathway (*Aro*), and in an operon important to *Salmonella* virulence (*PhoP/Q*). As a result, the bacteria appear to be extremely attenuated; doses as high as 1×10^9 cfu appear to be very safe. Other vaccines under development, such as CVD 908, have caused some systemic symptoms, e.g., fever or bacteremia, at doses as low as 1×10^7 cfu.

In addition to the *phoP/phoQ* deletion and the AroA-mutation, the bacterial cells of the invention may also contain a histidine mutation to further alternate virulence, although absence of the histidine mutation may improve immunogenicity. The bacterial cells of the invention are the most promising vaccine candidates to date because they are strongly immunogenic and safe, i.e., extremely attenuated.

EXAMPLE 1: Construction of vaccine strain

The bacterial cells of the invention were made by deleting approximately 1 kb of DNA in the *phoP/phoQ* regulon.

PhoP/phoQ deleted suicide vectors were constructed using methods known in the art. A DNA fragment containing the *phoP/phoQ* locus was obtained by PCR using wild type *S. typhimurium* chromosomal DNA as a template. PCR primers flanking the *phoP/phoQ* locus were engineered to contain terminal restriction enzyme recognition sites, e.g., recognition site for EcoRI, to facilitate subsequent cloning. Following amplification, the PCR product was digested with EcoRI and cloned into the EcoRI site in the polylinker of a high copy vector. The plasmid containing the *phoP/phoQ* DNA fragment was named pLH356.

Sequence analysis and restriction mapping of the

phoP/phoQ locus revealed four HpaI sites within the locus; no HpaI sites were found in the vector. To create an internal deletion within the *phoP/phoQ* locus, pLH356 DNA was cut to completion with HpaI, and religated, to yield with an internal deletion from nucleotides 376-1322 (pLH418). This deletion was confirmed by restriction digestion of the plasmid.

A DNA fragment containing the internally deleted *phoP/phoQ* locus was excised from pLH418 using the SacI/SphI restriction sites within the polylinker region of the vector. This fragment was cloned into compatible sites in the plasmid CVD442, which carries the *sacB* gene to allow positive selection for allelic exchange. The resulting suicide vector was called pLH423.

pLH423 was transformed into *E. coli lambda pir* SY327, and subsequently into *E. coli lambda pir* SM10 (strain LH425). *E. coli* strain LH425 was mated with *S. typhimurium* strain CS019. Single recombinants carrying plasmid sequences integrated onto the *S. typhimurium* chromosome were selected by plating on agar containing ampicillin and chloramphenicol (Strain LH428). These strains were confirmed to be ampicillin resistant and sucrose sensitive, i.e., death on 20% sucrose plates containing no NaCl when incubated at 30°C. These data confirm the integration of plasmid sequences into the *Salmonella* chromosome.

A P22 bacteriophage lysate was made from strain LH428; phage particles were concentrated 20× by high speed centrifugation and transduced into *S. typhi* strain 522Ty2 (a strain with a deletion in the *aroA* gene, and the G646 mutation which renders the organism auxotrophic for histidine). Single recombinant *S. typhi* organisms were selected by plating on LB plates supplemented with aromatic amino acids, cystine, histidine, and ampicillin (strain LH435).

Strain LH453 was grown with aromatic amino acids, cystine, and histidine (but without ampicillin) to mid logarithmic growth phase. Serial dilutions were plated on LB 20% sucrose plates lacking NaCl, and on LB plates lacking NaCl. The number of bacteria that grew on plates without sucrose was greater than the number that grew on sucrose-supplemented plates by a factor of three logs. These data suggest that many colonies lost plasmid sequences containing the sacB gene.

Multiple colonies from the sucrose selection were picked and confirmed to be ampicillin sensitive and sucrose resistant. Chromosomal DNA from approximately 10 colonies was purified and subjected to Southern blot analysis, utilizing the 2.3 kb fragment of wild type *phoP/phoQ* as a probe.

Southern blotting revealed the loss of two HpaI sites and an XmnI site known to be within the 1 kb deleted fragment of *phoP/phoQ* in several strains. One of these strains was designated TyLH445.

EXAMPLE 2: *in vitro* evaluation of TyLH445

TyLH445 was extensively characterized *in vitro* using standard clinical microbiological tests. The nutritional requirements of TyLH445 were evaluated. TyLH445 did not grow on M-9 plates unless supplemented with aromatic amino acid mix, cystine (*S. typhi* grows better with cystine), and histidine. These data confirmed that TyLH445 was AroA-, His-.

TyLH445 was found to agglutinate with polyclonal serum against *Salmonella* and polyclonal serum against *S. typhi Vi* antigen. Group D agglutination was found to be variable, perhaps due to excess Vi antigen. TyLH445 was also found to be indole negative (as are all *Salmonellae)*, and to produce very little hydrogen sulfide (as do many *S. typhi)*. Biochemical testing utilizing

both the VITEK system as well as the BBL Crystal Enteric organism identification system was also carried out. These data indicated that the TyLH445 strain was *S. typhi*.

Growth characteristics of TyLH445 were also evaluated. TyLH445 was found to grow just as quickly as its parent, 522Ty2, (*phoP/phoQ* locus intact). Growth *in vitro* was measured in aromatic amino acid/histidine/cystine-supplemented Luria broth at 37°C. Growth curves of the parent and vaccine strain were found to be essentially identical (see Fig. 10).

Standardized clinical testing methods were use to determine antibiotic sensitivity. TyLH445 and the parent strain, 522Ty2, were found to be sensitive to ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin, aminoglycosides, and third generation cephalosporins. No difference in zone sizes was detected between the parent and vaccine strains, suggesting that no other antibiotic resistance mechanisms, e.g., modification of antibiotic transport systems, or modification of the cell wall of the bacterium, were affected by introduction of the mutated *phoP/phoQ* locus into *S. typhi*.

The *phoP/phoQ* HpaI deletion mutants were tested for defensin sensitivity, a phenotype of *PhoP* null mutants. Defensin sensitivity assays were performed as follows.

Liquid cultures of strains to be tested were grown overnight. Cultures were then diluted 1:200, and grown to an optical density (OD_{600}) of approximately 0.2, after which the cells were diluted to concentration of approximately 1×10^5 organisms per 0.05 ml.

Two reactions were carried out for each strain: (1) vehicle alone (0.01% acetic acid in sterile water) and (2) defensin NP-1 solution (70 ug/ml in 0.01% acetic acid). An equal volume of bacterial suspension in

tryptone was added and the test tubes were incubated on a roller at 37°C for 2 hours. The final volume in each reaction tube was 0.1 ml, making the final concentration of defensin 35 ug/ml.

Defensin is inactivated by the high salt and high protein concentration present in bacterial growth media, e.g. LB broth. Thus, defensin activity was stopped by adding 900 ul of Luria broth to each tube. Serial dilutions of each tube were plated and cfu/ml was determined for both the control tube and treatment tube for each strain. Results were expressed as log of bacteria killed for each strain. Typically, 1.0-1.5 log of wild type bacteria were killed. *PhoP* null mutants generally exhibit 2-4 logs of killing. Since strains with slower growth rates appear less susceptible to defensin killing, the growth rate of each strain tested in the defensin sensitivity assay was measured. Strains with similar growth rates were compared in the defensin sensitivity assay.

The HpaI deletion was evaluated both in an *S. typhimurium* background and in the *S. typhi* background. In both backgrounds, the deletion mutation conferred sensitivity to rabbit defensin NP-1 at a concentration of 35 ug/ml. See Fig. 11 and Fig. 13. The difference between *PhoP*+ and HpaI deleted *PhoP* null mutants was less pronounced in the *S. typhi* strain, an effect that may reflect the slower growth rate of the less hardy *S. typhi* strain compared to the *S. typhimurium* strain which lacks the additional auxotrophies.

The state of *phoP* activation in bacteria with the HpaI *phoP/phoQ* deletion was tested utilizing a *LacZ* recorder gene fused to *phoP*-activated gene B (*pagB*). Since the efficiency of transduction utilizing P22 in *S. typhi* is low, these studies were performed in *S. typhimurium* rather than *S. typhi*. *PhoP* activation was

found to be 40-60 Miller units (Miller et al., 1972, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp. 352-355) in the presence of an intact *phoP/phoQ* locus, and just barely detectable in strains with the HpaI deletion (3cfusee Fig. 12).

EXAMPLE 3: *In vivo* evaluation of *S. typhimurium* HpaI deleted strain

As *S. typhi* strains are not pathogenic for mice, the HpaI *phoP/phoQ* deletion mutation was evaluated in both wild type and *aroA- S. typhimurium*. Female BALB/c mice were injected intraperitoneally with various dilutions of *S. typhimurium* LH430, a wild type *S. typhimurium* carrying the HpaI deletion. The LD_{50} of this strain was determined to be between 8.2×10^5 and 8.2×10^6. (All mice receiving 8.2×10^5 cfu survived, and all receiving 8.2×10^6 died.) These data are consistent with the LD_{50} data obtained with strains harboring transposon insertions at the *phoP/phoQ* locus.

Immunogenicity of the HpaI *phoP/phoQ* deletion was evaluated in *S. typhimurium aroA::tet* (LH481), a strain comparable to the human vaccine strain. Mice were inoculated intraperitoneally with 2.3×10^5 and 2.3×10^6 cfu of LH481 (4 mice per vaccine dose), and challenged 30 days later with 30 × the LD_{50} of wild type organisms. All mice but one mouse survived. The mouse that died was in the group that received the lower vaccine dose. No animal receiving the higher vaccine dose became ill.

EXAMPLE 4: Phase I study human studies

The vaccine strain was administered to human volunteers at doses of 1×10^5 to 1×10^{10} cfu/single oral dose. Two volunteers received each dose; 3 volunteers were given a dose of 1×10^8 cfu/ml. Volunteers were evaluated at various time points following administration of the vaccine.

Safety

To detect the presence of the vaccine strain in patient blood, Bactec blood cultures were performed in duplicate on days 4, 6, 8, 10, 12 after taking vaccine. Bacteremia was not detected in any of the volunteers.

Thirteen adult human volunteers have received escalating single oral doses of this new attenuated typhoid fever vaccine. No individuals have had side effects of any sort. Specifically, there have been no fevers, no gastrointestinal symptoms, and no constitutional symptoms. Volunteers have been subjected to serial blood cultures on a preset schedule after receiving the oral vaccine 2 sets of BACTEC blood cultures performed on each of days 4, 6, 8, 10 and 12 after receiving the vaccine, and no positive blood cultures have been noted. Volunteers have been followed up at 2 months after receiving the vaccine, and no late symptoms have been reported.

Colonization

Stool samples were tested for the presence of the vaccine strain TyLH455 using methods known in the art. Primary stool was evaluated for the presence of the vaccine strain on culture plates. In some cases, it was necessary to enrich stool samples for the vaccine strain by incubating the stool overnight in BBL Selenite F broth supplemented with Aro/His/Cystine in order to detect the bacteria. This medium is somewhat inhibitory for *E. coli* and but promotes *Salmonella* growth.

Volunteers have been colonized for various time periods from 1-6 days after receiving the vaccine. With the highest doses (10^9 or 10^{10}) volunteers have had positive primary culture plates in the initial 1-3 days post vaccination, whereas at lower doses, only selenite enrichment broth cultures (selective medium for *Salmonella* which inhibits other enterics) have been

positive for the vaccine organism. No volunteer studied thus far has had prolonged carriage of the vaccine organism at 2 months of followup.

Table 17

Dose	Number	Colonization
10^5	2	NO
10^6	2	2/2 for 1-2 days
10^7	2	1/2 for 3 days
10^8	3	1/3 for 6 days
10^9	2	2/2 for 4-6 days both had positive primary plates day 1
10^{10}**	2	2/2 for 3-6 days both had positive primary plates on days 1 and 2

* Measured by whole cell and LPS ELISAs and Widal test vs. H flagellar antigen. Sera analyzed at 1:40 and higher dilutions in all tests.

** One of these volunteers has received a booster dose of 10^{10} organisms, given one month after the primary inoculation (serologies pending).

Immunogenicity

Induction of an immune response to the vaccine strain was measured by standard ELISA assays. Sera was collected from volunteers 0, 7, 14, 21, and 28 days after receiving a single oral dose of the vaccine. ELISA assays were carried out using whole bacteria TyLH445 and *S. typhi* LPS (SIGMA, St. Louis, MO) as antigens. Day 0 serum from each volunteer was used as an internal negative control. Convalescent sera from patients previously infected with wild type *S. typhi* (most from

Mexico) were used as positive controls.

Several volunteers had documented seroconversion at 21 days after receiving the vaccine, as measured by ELISA in which IgG antibodies directed against whole vaccine organisms or against *S. typhi* LPS were detected. Sera taken from patients prior to administration of the vaccine (pre-immune sera) were tested, and the data used to establish a baseline. Patient sera taken at various time points after vaccination were considered positive if the test results were 0.2 ELISA OD units greater than that of the preimmune serum.

Other Embodiments

Other embodiments, e.g., strains of *Salmonella* which contain only a deletion in the phoP/phoQ regulatory locus to attenuate virulence, and strains which, in addition to a phoP related mutation or genetic alteration, also contain an attenuating mutation in another gene, e.g., cya gene (adenylate cyclase) or crp gene (adenylate cyclase receptor), are also within the claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Miller, Samuel I.
Mekalanos, John J.

(ii) TITLE OF INVENTION: SALMONELLA VACCINES

(iii) NUMBER OF SEQUENCES: 15

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 and WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/090,526
(B) FILING DATE: July 9, 1993

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Clark, Paul T.
(B) REGISTRATION NUMBER: 30,162
(C) REFERENCE/DOCKET NUMBER: 00786/220001

(ix) TELECOMMUNICATION INFORMATION:
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(C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	2320
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	double
(D)	TOPOLOGY:	linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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TATATGCGTG AGAAAAATTA GCATTCAAAT CTATAAAAGT TAGATGACAT TGTAGAACCG   540

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ATCAGGAGCG ATAGCGGTGA ATTATTCGTG GTTTTGTCGA TTCGGCATAG TGGCGATAAC   660

TGAATGCCGG ATCGGTACTG CAGGTGTTTA AACACACCGT AAATAATAAG TAGTATTAAG   720

GAGTTGTT                                                            728

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Met Lys Asn Ile Ile Leu Ser Thr Leu Val Ile Thr Thr Ser Val Leu
                  5                  10                  15

GTT GTA AAT GTT GCA CAG GCC GAT ACT AAC GCC TTT TCC GTG GGG TAT      824
Val Val Asn Val Ala Gln Ala Asp Thr Asn Ala Phe Ser Val Gly Tyr
             20                  25                  30

GCA CGG TAT GCA CAA AGT AAA GTT CAG GAT TTC AAA AAT ATC CGA GGG      872
Ala Arg Tyr Ala Gln Ser Lys Val Gln Asp Phe Lys Asn Ile Arg Gly
         35                  40                  45

GTA AAT GTG AAA TAC CGT TAT GAG GAT GAC TCT CCG GTA AGT TTT ATT      920
Val Asn Val Lys Tyr Arg Tyr Glu Asp Asp Ser Pro Val Ser Phe Ile
     50                  55                  60

TCC TCG CTA AGT TAC TTA TAT GGA GAC AGA CAG GCT TCC GGG TCT GTT      968
Ser Ser Leu Ser Tyr Leu Tyr Gly Asp Arg Gln Ala Ser Gly Ser Val
65                  70                  75                  80
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GAG CCT GAA GGT ATT CAT TAC CAT GAC AAG TTT GAG GTG AAG TAC GGT    1016
Glu Pro Glu Gly Ile His Tyr His Asp Lys Phe Glu Val Lys Try Gly
                 85                  90                  95

TCT TTA ATG GTT GGG CCA GCC TAT CGA TTG TCT GAC AAT TTT TCG TTA    1064
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TAC GCG CTG GCG GGT GTC GGC ACG GTA AAG GCG ACA TTT AAA GAA CAT    1112
Tyr Ala Leu Ala Gly Val Gly Thr Val Lys Ala Thr Phe Lys Glu His
        115                 120                 125

TCC ACT CAG GAT GGC GAT TCT TTT TCT AAC AAA ATT TCC TCA AGG AAA    1160
Ser Thr Gln Asp Gly Asp Ser Phe Ser Asn Lys Ile Ser Ser Arg Lys
    130                 135                 140

ACG GGA TTT GCC TGG GGC GCG GGT GTA CAG ATG AAT CCG CTG GAG AAT    1208
Thr Gly Phe Ala Trp Gly Ala Gly Val Gln Met Asn Pro Leu Glu Asn
145                 150                 155                 160

ATC GTC GTC GAT GTT GGG TAT GAA GGA AGC AAC ATC TCC TCT ACA AAA    1256
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Ile Asn Gly Phe Asn Val Gly Val Gly Tyr Arg Phe
            180                 185

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GATTCACTCT GAAAAATTTT CCTGGAATTA ATCACAATGT CATCAAGATT TTGTGACCGC  1600

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ATTACGTGAC AAGATAGTCA TTTATAAAAA ATGCACAAAA ATGTTATTGT CTTTTATTAC  1960

TTGTGAGTTG TAGATTTTTC TTATGCGGTG AATCCCCCTT TGCGGCGGGG CGTCCAGTCA  2020

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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	53
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	688
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	double
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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GAAATTATAC TCCATGAGCT GAAAGAAGGA AATTCTGAAT CTCGTTCGGT GCAATTAAAT 420

ACGCCAATAC AGGTCGGTGA ATTGCTTATC CTGATTCGCC CGGAAAGCGA GCCGTGGGTG 480

CCCGAGCAGC CTGAGAAGTT AGAAACGTCT GCAAAAAAGA ACGAGCCGCG TTTTAAAAAC 540

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TGGATACTTA ACTCGCCGCA GCGGCAGGCC CGAGAGCTCG ATTCGTTATT GGGGCAGGAG 660

AAGGAGCGTT TTCAGGTGTT GCCAGGCC 688

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AATATCGCCC TGAGCA 16

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4044
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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ATTGATATGA GCAGGAATAT CGAT 4044

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

(i) SEQUENCE CHARACTERISTICS:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 87 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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His Val Leu Ala Ser Ser Gly His Cys Ser Gly Leu Gln Gln Ala Ser
            20                  25                  30

Trp Asp Ile Phe Ile Tyr Asp Phe Gly Ser Lys Thr Pro Gln Pro Pro
        35                  40                  45

Thr Asn Thr Asp Lys Lys Gln Ala Arg Gln Ile Ser Ser Pro Ser Cys
    50                  55                  60

Pro Thr Thr Lys Pro Met Met Ser Ala Pro Val Asn Asp Ala Arg Lys
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Gly Asn Thr Phe Ser Arg Thr
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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 178 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7

```
Met Thr Leu Leu Ser Gly Lys Thr Thr Leu Val Leu Cys Leu Ser Ser
1               5                  10                  15

Ile Leu Cys Gly Cys Thr Thr Asn Gly Leu Pro Thr Pro Tyr Ser Ile
            20                  25                  30

Asn Leu Ser Phe Pro Val Ile Thr Gln Asn Gln Ile Asn Ser Gly Gly
        35                  40                  45

Tyr Tyr Ile Asn Asp Ala Glu Gln Ile Arg Thr Thr Asp Gly Leu Cys
    50                  55                  60

Leu Asp Ala Gly Pro Asp Gln Gln Asn Arg Leu Thr Leu Arg Glu Cys
65                  70                  75                  80

Lys His Val Gln Ser Gln Leu Phe Ser Phe His Arg Asp Arg Ile Thr
                85                  90                  95

Gln Gly Glu Lys Cys Leu Asp Ala Ala Asp Lys Val Gln Lys Lys Ala
            100                 105                 110

His Gln Ser Phe Phe Ile His Ala Arg Val Met Ile Thr Ser Ala Gly
        115                 120                 125

Ser Leu Ile Ile Thr Lys Leu Arg Gly Asn Arg Ala Glu Asn Ala Trp
    130                 135                 140

Ala Gln Ile Ala Leu Leu Ser Glu Lys Ala Thr Leu Leu Cys Trp Pro
145                 150                 155                 160
```

Ile Ala Ile Leu Val Ala Pro Trp Asn Leu Pro Ser Gly Ser Arg Thr
165 170 175

Pro Leu

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 79 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8

Met Phe Val Glu Leu Val Tyr Asp Lys Arg Asn Val Glu Gly Leu Pro
1 5 10 15

Gly Ala Arg Glu Ile Ile Leu Asn Glu Leu Thr Lys Arg Val His Gln
20 25 30

Leu Phe Pro Asp Ala Gln Val Lys Val Lys Pro Met Gln Ala Asn Ala
35 40 45

Leu Asn Ser Asp Cys Thr Lys Thr Glu Lys Glu Arg Leu His Arg Met
50 55 60

Leu Glu Glu Met Phe Glu Glu Ala Asp Met Trp Leu Val Ala Glu
65 70 75

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 246 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9

Met Asn Lys Ile His Val Thr Tyr Lys Asn Leu Leu Leu Pro Ile Thr
1 5 10 15

Phe Ile Ala Ala Thr Leu Ile Ser Ala Cys Asp Asn Asp Lys Asp Ala
20 25 30

Met Ala Glu Ala Glu Lys Asn Gln Glu Lys Tyr Met Gln Lys Ile Gln
35 40 45

Gln Lys Glu His Gln Gln Ser Met Phe Phe Tyr Asp Lys Ala Glu Met
50 55 60

Gln Lys Ala Ile Ala Asn Ile Asn Ala Lys Gly Gly Ala Asn Leu Ala
65 70 75 80

Ile Ile Glu Val Arg Phe Phe Lys Gly Gly Tyr Ser Phe Ile Arg Gln
85 90 95

Ser Val Asn Thr Pro Ala Lys Val Glu Val Phe Lys Phe Asn Asn Gly
100 105 110

Tyr Trp Gly Gly Pro Ser Pro Val Asn Leu Thr Ile Phe Gly Thr Ile
115 120 125

Thr Glu Glu Gln Lys Gln Glu Ala Leu Lys Glu Ala Leu Phe Lys Phe
130 135 140

Asp Ser Ile Asn Phe Ser Ile Ile Pro Glu Arg Ile Gln Glu Thr Ile
145 150 155 160

Lys Arg Ala Asn Ala Ser Gly Ile Ile Ser Val Thr Glu Asp Ser Asp
165 170 175

Ile Val Val Arg Ala Glu Ile Ala His Asn Gly Glu Phe Val Tyr Asp
180 185 190

Ile Thr Ile Thr Ala Lys Asn Thr Ala Arg Ala Val Met Thr Leu Asn
195 200 205

Lys Asp Gly Ser Ile Ala Gly Tyr Glu Ile Lys Glu Pro Phe Ala Pro
210 215 220

Lys Lys Glu Ala Glu Lys Ala Gln Gln Leu Val Glu Gln Ser Arg Lys
225 230 235 240

Asp Ile Glu Ser Pro Ala
245

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3700
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10

TTTTGGTTTG CTGCCGTTTG GGATAACTGC ATAGAGAGCG GCCAAGTCGC TTGCGGTCGG 60

TATCTCGAGT ATATCGAAAT CCATGTGGCC ATTGACCTCT TCAAGCGCTC ACGTTAACTA 120

CCTGCTCTTT TTTGAGCACC AACATCCCAG GTTCGTCACA GTAAATCGTA TCGTGATTAT 180

TGCTAATCGT CAGTTTACCG CTCCGAAAGC AAACTAAAGT GAAACTGCTT ACATAAAGAT 240

TTTTGATGGT AACCTGCTGA GTCTGACTTT TAATTTGCTG CCGGGTATTT GTCAAAAGTG 300

ATTTTAATTT CTGTAAGTTA TCTGCGGCAG GACGCTGATG ACTATTACTT ACAAAGGTTA 360

CATTTTCCAT ATTATCCCTT TGTTGAACTT ATTTTAATGT TCCTTACTGG TATCCTACTG 420

AAAAAATCTG AGTTGTAAAT GCTCTTTATT AGCGTGTGTT GGCAATGGTC TGATTGTTAC 480

ACCAAAAGAA CCCAAATTTG GGTAATTTAT CTACAGTAGT TTAAGCCCCA ATGGGGATGA 540

TGGTTCTTTT AATATGTGTT GAGACGCATT ATACAGAATA AATTGATTTT ATTTCTCACT 600

TTTCATTCTA TTTTCATCAG GAATCCCTGT GTCCTGTGCG GTAATCTGCT GCTATCGAGG 660

AACGACAGAC ATCGCTAACA GTATATATGG AAACATCAAA AGAGAAGACG ATAACAAGCT 720

TTCCAGGGCC ATACATAGTT CGATTACTTA ACAGCTCACT GAACGGCTGT GAGTTTCCAT 780

TGGGCCTGAC AGGCCGAACA CTCTTTGTGG TAGGTCAGAG TGATGCGCTC ACTGCTTCAG 840

GTCAATGTGA TAGCTCCCTG ATATACCTGC CGATAGCTTT TTTATCCCGC TGGACCATGG 900

CGGAGTAAAT TTTAGGGAAA TCCAGGTGGA TACGGATGCG ACCGAAATTA TACTCCATGA 960

GCTGAAAGAA GGAAATTATG TCTGAATCTC GTTCGGTGCA ATTAAATACG CCAATACAGG 1020

TCGGTGAATT GCTTATCCTG TGATTCGCCC GGAAAGCGAG CCGTGGGTGC CCGAGCAGCC 1080

TGAGAAGTTA GAAACGTCTG CATAAAAAAG AACGAGCCGC GTTTTAAAAA CGGAATTGTA 1140

GCAGCACTGG CCGGGTTTTT TATAGAAAGT TGGGAATTGG GACTGTGGGG ACGTTATGGA 1200

TACTTAACTC GCCGCAGCGG CAGGCCGCAG GTGTAAGAGC TCGATTCGTT ATTGGGGCAG 1260

GAGAAGGAGC GTTTTCAGGT GTTGCCAGGC CGGGACGGAA AATGCTCTAT GTCGCTGCGC 1320

AAAATGAAAG AGATACGTTG TGGGCTCGTC AGGTTTTAAA TAGCGAGGGG CGATTATGAT 1380

AAAAATGCGC GAGTGATTAA CGAAAACGAA GAAAATAAGC GTAGAATCTC TATCTGGCTG 1440

GATACCTATT ATCCGCAGCT GGCTTATTAT CGGATTCATT TCGATTAGAG CCGCGTAAAC 1500

CCGTTTTCTG GCTAAGCCGC CAGCGAAACA CGATGAGCAA GAAAGAGTCT CGAGGTGTTA 1560

AGTCAAAAGC TGAGAGCGCT AATGCCTTAC GCGGATTCGG TTAACATCAA ACGTTGATGG 1620

ACGATGTTAC CGCAGCAGGC CAGGCGGAAG CGGGGCTAAA ACAGCAGGCG TTAAGAAGAT 1680

TACCTTATTC CCGCAGGAAT CATAAGGGGG GCGTAACGTT TGTTATTCAG GGGGCGCTCG 1740

GTGAGATGAT GTAGAAATAC TCAGAGCCCG TCAATTTGTC GATAGCTATT ACCGCACATG 1800

GGGAATGGGA CGCTATGTGC AGTTTGCGAT CGAATTAAAA GATGACTGGC TCAAGGGGCG 1860

CTCATTTGAG CAGTACGGGG CGGAAGGTTA TATCAAAATG AGCCCAGGCC ATTGGTATTT 1920

CCCAAGCCCA GAGGGCTTTA ATTTAACGTA AATAAGGAAG TCATTATGGC AACACCTTGG 1980

TCAGGCTATC TGGATATGGA CGTCTCAGCA AAATTTGATA CGGGCGTTGA TAATCTACAA 2040

ACGCAGGTAA CAGAGGCGAT GTTACTGGAT AAATTAGCAG CAAAACCCTC CGATCCGGCG 2100

CTACTGGCGG CGTATCAGAG TAAGAAAAAC TCTCGGAATA TAACTTGTAC CGTAACGCGC 2160

AATCGAACAC GGTAAAAGTC TTTAAGGATA TGATTGATGC TGCCATTATT CAGAACTTCC 2220

GTTAATCAGT TATAAGGTGG ATTATGTCGA TTAAGCAACT ATTGTCCCTG AGAATGCCGT 2280

TATAGGGCAG GCGGTCAATA TCAGGTCTAT GGAAATAGAA CGGACATTGT CTCGCTGGAT 2340

GACCGGCTAC TCCAGGCTTT TTCTGGTTCG GCGATTGCCT AGAAACGGCT GTGGATAAAC 2400

AGACGATTAC CAACAGGATT GAGGACCCTA ATCTGGTGAC GGATTATTTC CTAAAGAGCT 2460

GGCTATTTCG CAAGAGATGA TTTCAGATTA TAACCTGTAT GTTTCTATGA GGTCAGTACC 2520

CTTACTCGTA AAGGAGTCGG GGCTGTTGAA ACGCTATTAC GCTCATGATT CTTGGATGTC 2580

GATATCTATA TACTTTTCTG CTGGTAATGA CCCTTGCCGG CTGTAAGGAT AAGGATCTTA 2640

GCTTTTAAAA GGACTGGACC AGGAACAGGC TAATGAGGTC ATTGCCGTTC TGCAAATGCA 2700

CAGAAATATA GAGGCGAATA AAATTGATAG CGGAAAATTG GGCTATAGCA TTACCGTTGC 2760

TGAGCAGGTA CTGATTTTAC CGCTGCGGTG TACTGGATTA AAACTTATCA GCTTCCTCCC 2820

CGGCCACGGG TAATTGGAAA TAGCGCAGAT GTTCCCGGCG GATTCGCTGG TATCGTCTCC 2880

GCGAGCTGAA AAGGAAAACC AGGTTATATT CGGCTATTGA ACAGCGACTG GAACAGTCAT 2940

TACAGACGAT GGAGGGCGAT GTGCTCTCCG CCAGGGTCCA TATTAGTTAT GATATTGATG 3000

CTGGTGAAAA TGGCCGCCCG CAAGGCAAAA CCTGTTCATC TGTCGGCATT AGCCGTATAT 3060

GAACGAGGTT CGCCGCTTGC GCATCAAGAA GATCAGCGAT ATCAAGCGTT TCTTAAAGAA 3120

TAGTTTTGCC GATGTGGATT ATGACAACAA TTTCTGTTGT GTTGTCAGAA CGTTCTGATG 3180

CCCAATTACA GGCTCCCGGC ACACCAGTAA AAGTAACGTA ATTCTTTTGC AACCAGTTGG 3240

ATTGTTTTGA TTATTTTGTT ATCCGTGATG TCAGATACAG GCTTTGGCGT CTGGTATTAC 3300

AAAAACCATT ATGCCCGCAA TAAGAAAGGC ATAACGGGGA GTACTGATGA TAAGGCGAAA 3360

TCGTCAAATG AATAGGCAGC CATTACCCAT TATCTGGCAA AGAATCATTT TTGATCCGTT 3420

ATCGTATATC CATCCTCAGC GGTTGCAGAT AGCGCCGGAA ATGATTGTCA GACCGCGCCA 3480

CGCGAAATGA GTTAATACTG GCGGCATGGC GGCGGCTTAA GAACGGAGAA AAGGAGTGTA 3540

TTCAAAACTC ACTGACGCAG CTGTGGCTGC TCAGTGGCGC CGACTGCCGC AAGTAGCGTA 3600

TTTACTAAAC TGAGAGCCGA TCTGGCAAGG CAGGGAGCCT TGCTTGGCCT AGCCGGATTG 3660

GGCGAAATGA GTTAATACTG GCGGCATGGC GGCTTGCCAT 3700

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	392 amino acids
(B) TYPE:	amino acid
(D) TOPOLOGY:	linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11

```
Met Glu Thr Ser Lys Glu Lys Thr Ile Thr Ser Pro Gly Pro Tyr Ile
 1               5                  10                  15

Val Arg Leu Leu Asn Ser Ser Leu Asn Gly Cys Glu Phe Pro Leu Leu
            20                  25                  30

Thr Gly Arg Thr Leu Phe Val Val Gly Gln Ser Asp Ala Leu Thr Ala
        35                  40                  45

Ser Gly Gln Leu Pro Asp Ile Pro Ala Asp Ser Phe Phe Ile Pro Leu
    50                  55                  60

Asp His Gly Gly Val Asn Phe Glu Ile Gln Val Asp Thr Asp Ala Thr
65                  70                  75                  80

Glu Ile Ile Leu His Glu Leu Lys Glu Gly Asn Ser Glu Ser Arg Ser
                85                  90                  95

Val Gln Leu Asn Thr Pro Ile Gln Val Gly Glu Leu Leu Ile Leu Ile
            100                 105                 110

Arg Pro Glu Ser Glu Pro Trp Val Pro Glu Gln Pro Glu Lys Leu Glu
        115                 120                 125

Thr Ser Ala Lys Lys Asn Glu Pro Arg Phe Lys Asn Gly Ile Val Ala
    130                 135                 140

Ala Leu Ala Gly Phe Phe Ile Leu Gly Ile Gly Thr Val Gly Thr Leu
145                 150                 155                 160

Trp Ile Leu Asn Ser Pro Gln Arg Gln Ala Ala Glu Leu Asp Ser Leu
                165                 170                 175

Leu Gly Gln Glu Lys Glu Arg Phe Gln Val Leu Pro Gly Arg Asp Lys
            180                 185                 190

Met Leu Tyr Val Ala Ala Gln Asn Glu Arg Asp Thr Leu Trp Ala Arg
        195                 200                 205

Gln Val Leu Ala Arg Gly Asp Tyr Asp Lys Asn Ala Arg Val Ile Asn
    210                 215                 220

Glu Asn Glu Glu Asn Lys Arg Ile Ser Ile Trp Leu Asp Thr Tyr Tyr
225                 230                 235                 240

Pro Gln Leu Ala Tyr Tyr Arg Ile His Phe Asp Glu Pro Arg Lys Pro
                245                 250                 255

Val Phe Trp Leu Ser Arg Gln Arg Asn Thr Met Ser Lys Lys Glu Leu
            260                 265                 270

Glu Val Leu Ser Gln Lys Leu Arg Ala Leu Met Pro Tyr Ala Asp Ser
        275                 280                 285

Val Asn Ile Thr Leu Met Asp Asp Val Thr Ala Ala Gly Gln Ala Glu
    290                 295                 300
```

```
Ala Gly Leu Lys Gln Gln Ala Leu Pro Tyr Ser Arg Arg Asn His Lys
305                 310                 315                 320

Gly Gly Val Thr Phe Val Ile Gln Gly Ala Leu Asp Asp Val Glu Ile
                325                 330                 335

Leu Arg Ala Arg Gln Phe Val Asp Ser Tyr Tyr Arg Thr Trp Gly Gly
            340                 345                 350

Arg Tyr Val Gln Phe Ala Ile Glu Leu Lys Asp Asp Trp Leu Lys Gly
        355                 360                 365

Arg Ser Phe Gln Tyr Gly Ala Glu Gly Tyr Ile Lys Met Ser Pro Gly
    370                 375                 380

His Trp Tyr Phe Pro Ser Pro Leu
385                 390
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 80 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12

```
Met Ala Thr Pro Trp Ser Gly Tyr Leu Asp Asp Val Ser Ala Lys Phe
  1               5                  10                  15

Asp Thr Gly Val Asp Asn Leu Gln Thr Gln Val Thr Glu Ala Leu Asp
            20                  25                  30

Lys Leu Ala Ala Lys Pro Ser Asp Pro Ala Leu Leu Ala Ala Tyr Gln
        35                  40                  45

Ser Lys Leu Ser Glu Tyr Asn Leu Tyr Arg Asn Ala Gln Ser Asn Thr
    50                  55                  60

Val Lys Val Phe Lys Asp Ile Asp Ala Ala Ile Ile Gln Asn Phe Arg
65                  70                  75                  80
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 101 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13

```
Met Ser Ile Ala Thr Ile Val Pro Glu Asn Ala Val Ile Gly Gln Ala
 1               5                  10                  15

Val Asn Ile Arg Ser Met Glu Thr Asp Ile Val Ser Leu Asp Asp Arg
            20                  25                  30

Leu Leu Gln Ala Phe Ser Gly Ser Ala Ile Ala Thr Ala Val Asp Lys
        35                  40                  45

Gln Thr Ile Thr Asn Arg Ile Glu Asp Pro Asn Leu Val Thr Asp Pro
    50                  55                  60

Lys Glu Leu Ala Ile Ser Gln Glu Met Ile Ser Asp Tyr Asn Leu Tyr
65                  70                  75                  80

Val Ser Met Val Ser Thr Leu Thr Arg Lys Gly Val Gly Ala Val Glu
                85                  90                  95

Thr Leu Leu Arg Ser
            100
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 252 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14

```
Met Ile Arg Arg Tyr Leu Tyr Thr Phe Leu Leu Val Met Thr Leu Ala
 1               5                  10                  15

Gly Cys Lys Asp Lys Asp Leu Leu Lys Gly Leu Asp Gln Glu Gln Ala
            20                  25                  30

Asn Glu Val Ile Ala Val Leu Gln Met His Asn Ile Glu Ala Asn Lys
        35                  40                  45

Ile Asp Ser Gly Lys Leu Gly Tyr Ser Ile Thr Val Ala Glu Pro Asp
    50                  55                  60

Phe Thr Ala Ala Val Tyr Trp Ile Lys Thr Tyr Gln Leu Pro Pro Arg
65                  70                  75                  80

Pro Arg Val Glu Ile Ala Gln Met Phe Pro Ala Asp Ser Leu Val Ser
                85                  90                  95

Ser Pro Arg Ala Glu Lys Ala Arg Leu Tyr Ser Ala Ile Glu Gln Arg
            100                 105                 110
```

Leu Glu Gln Ser Leu Gln Thr Met Glu Gly Val Leu Ser Ala Arg Val
115 120 125

His Ile Ser Tyr Asp Ile Asp Ala Gly Glu Asn Gly Arg Pro Pro Lys
130 135 140

Pro Val His Leu Ser Ala Leu Ala Val Tyr Glu Arg Gly Ser Pro Leu
145 150 155 160

Ala His Gln Ile Ser Asp Ile Lys Arg Phe Leu Lys Asn Ser Phe Ala
165 170 175

Asp Val Asp Tyr Asp Asn Ile Ser Val Val Leu Ser Glu Arg Ser Asp
180 185 190

Ala Gln Leu Gln Ala Pro Gly Thr Pro Val Lys Arg Asn Ser Phe Ala
195 200 205

Thr Ser Trp Ile Val Leu Ile Ile Leu Leu Ser Val Met Ser Ala Gly
210 215 220

Phe Gly Val Trp Tyr Tyr Lys Asn His Tyr Ala Arg Asn Lys Lys Gly
225 230 235 240

Ile Thr Ala Asp Asp Lys Ala Lys Ser Ser Asn Glu
245 250

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 818
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CATAACAACT CCTTAATACT ACTTATTATT TACGGTGTGT TTAAACACCT GCAGTACCGA 60

TCCGGCATTC AGTTATCGCC ACTATGCCGA ATCGACAAAA CCACGAATAA TTCACCGCTA 120

TCGCTCCTGA TGTGTTTACT TCCTGAAAGA TATTTTTACT ACCGAAGCAC TCTATCGCTC 180

ATTTAGGTAA CCGGTTCTAC AATGTCATCT AACTTTTATA GATTTGAATG CTAATTTTTC 240

TCACGCATAT ATATTTAACA GAAACCATAA AGTGTTTAGC CACTATAGAA CAACAAATCA 300

CCCATGCAAC ATTTTGATAT TTAAAGAGAA AATCTCACAA CCACATTAAG AAACTTGACA 360

CCGTTCGGCT AAAAAACATG TCATTAAGCA AACTCGCCAT ATAATCAGAA CATATCGCAT 420

TGTGCTTCAC AGTCCTCACG TGACGCTCCA TCCGCAATAC GGTTATATGC CATCGCAGGC 480

GCTGTAATCA TATTCACGAT GATGCTTAGC ACGCTTTATT CCCGCTCCGA TTTAATCTTT 540

TAATATATCT ATCAGTTACA ACATTTCTTG TTATATTATA AGAATAGAAT CAACACCACA 600

ATTCCAACAT AAATATCACC TGTGTTTAGA GAGAATTTAC ATTCCAAAAA AATAATAACT 660

```
AACGCAAATA TTGAACACGC GATAAAAAAG TCTATTTCGC TATAAAACCC ATTATTATTA   720
AGAGTGGTTA ACTCTTCGTT GAATAAAAAA TGTCAATGAC GTTCCATAAT TCAGGAGATG   780
AACTTCACAA GTCATTATAT ATAACAGGAG GTGCTATG                           818
```

TABLE 12

Bacterial strains.

Strain	Genotype	Source
S. typhimurium		
14082s	Wild type	ATCC
CS019	*phoN2 zxx*::6251Tn*10*d-Cm	25
CS015	*phoP-102*::Tn*10*d-Cm	25
AD154	*phoP12 purB1744*::Tn10	3
TT13208	*phoP105*::Tn*10d*	26
CS585	*pagD1*::Tn*phoA phoN2 zxx*::6215Tn*10*d-Cm	This study
CS586	*pagD1*::Tn*phoA phoP105*::Tn*10*d *phoN2 zxx*::6215Tn*10*d-Cm	This study
CS619	*pagE1*::Tn*phoA phoN2 zxx*::6215Tn*10*d-Cm	This study
CS620	*pagE1*::Tn*phoA phoP105*::Tn*10*d *phoN2 zxx*::6215Tn*10*d-Cm	This study
CS1599	*pagF1*::Tn*phoA phoN2 zxx*::6215Tn*10*d-Cm	This study
CS1600	*pagF1*::Tn*phoA phoP105*::Tn*10*d *phoN2 zxx*::6215Tn*10*d-Cm	This study
CS334	*pagG1*::Tn*phoA phoN2 zxx*::6215Tn*10*d-Cm	This study
CS335	*pagG1*::Tn*phoA phoP105*::Tn*10*d *phoN2 zxx*::6215Tn*10*d-Cm	This study
CS1488	*pagH1*::Tn*phoA phoN2 zxx*::6215Tn*10*d-Cm	This study
CS1489	*pagH1*::Tn*phoA phoP105*::Tn*10*d *phoN2 zxx*::6215Tn*10*d-Cm	This study
CS2054	*pagI1*::Tn*phoA phoN2 zxx*::6215Tn*10*d-Cm	This study
CS2055	*pagI1*::Tn*phoA phoP105*::Tn*10*d *phoN2* zxx::6215Tn*10*d-Cm	This study
CS1074	*pagJ1*::Tn*phoA phoN2* zxx::6215Tn*10*d-Cm	This study
CS1075	*pagJ1*::Tn*phoA phoP105*::Tn*10*d *phoN2* zxx::6215Tn*10*d-Cm	This study
CS767	*pagK1*::Tn*phoA phoN2* zxx::6215Tn*10*d-Cm	This study
CS768	*pagK1*::Tn*phoA phoP105*::Tn*10*d *phoN2* zxx::6215Tn*10*d-Cm	This study
CS993	*pagL1*::Tn*phoA phoN2* zxx::6215Tn*10*d-Cm	This study
CS994	*pagL1*::Tn*phoA phoP105*::Tn*10*d *phoN2* zxx::6215Tn*10*d-Cm	This study
CS1845	*pagM1*::Tn*phoA phoN2* zxx::6215Tn*10*d-Cm	This study
CS1846	*pagM1*:Tn*phoA phoP105*::Tn*10*d *phoN2* zxx::6215Tn*10*d-Cm	This study
CS728	*pagN1*::Tn*phoA phoN2* zxx::6215Tn*10*d-Cm	This study
CS729	*pagN1*::Tn*phoA phoP105*::Tn*10*d *phoN2* zxx::6215Tn*10*d-Cm	This study
CS1194	*pagO1*::Tn*phoA phoN2* zxx::6215Tn*10*d-Cm	This study
CS1195	*pagO1*::Tn*phoA phoP105*::Tn*10*d *phoN2* zxx::6215Tn*10*d-Cm	This study
CS1247	*pagP1*::Tn*phoA phoN2* zxx::6215Tn*10*d-Cm	This study
CS1248	*pagP1*::Tn*phoA phoP105*::Tn*10*d *phoN2* zxx::6215Tn*10*d-Cm	This study
AK3011-3314	Collection of Randomly spaced Tn*10* Δ16Δ17 insertions	18
E. coli		
SM10(pRT291)	Contains plasmid pRT291 (Tn*phoA*) derived from pRK290 selecting for Tet^r and Km^r	37
MM294(pPH1JI)	Contains Gm^r plasmid pPH1JI, which is in incompatible with pRK290	37

3 Behlau et al., 1993, J. Bacteriol., 175:4475-84

18 Lehrer et al., 1991, Cell, 64:229-30

25 Miller et al., 1989, Proc. Natl. Acad. Sci. USA, 86:5054-58

26 Miller et al., 1990, J. Bacteriol., 172:2485-90

37 Taylor et al., 1989, J. Bacteriol., 171:1870-78

TABLE 13

Comparison of *pag*::*phoA* activity in strains with wild type and null *phoP*$^{-}$ loci.

Allele	Activity (Units of AP)[a]				
	Logarithmic growth		Stationary growth		Fold
	PhoP^{+}	PhoP^{-}	PhoP^{+}	PhoP^{-}	Reduction[b]
pagD1::Tn*phoA*	32	2	79	9	16
pagE1::Tn*phoA*	96	2	108	3	48
pagF1::Tn*phoA*	89	4	276	10	22
pagG1::Tn*phoA*	35	1	65	6	35
pagH1::Tn*phoA*	35	5	38	6	7
pagI1::Tn*phoA*	12	2	24	8	6
pagJ1::Tn*phoA*	123	8	944	88	15
pagK1::Tn*phoA*	30	3	123	26	10
pagL1::Tn*phoA*	7	1	35	4	7
pagM1::Tn*phoA*	92	11	439	130	8
pagN1::Tn*phoA*	23	1	58	2	23
pagO1::Tn*phoA*	31	2	54	12	16
pagP1::Tn*phoA*	38	1	27	3	38

[a] The AP activity values are presented in units as defined by Miller for ß-galactosidase (24). The values are representative of experiments (performed in duplicate) that were repeated on three separate occasions. PhoP^{+} denotes the *pag*::Tn*phoA* insertion in strain CS019 containing a wild type *phoP* locus. PhoP^{-} denotes an isogenic strains carrying the *phoP105*::Tn*10* allele.

[b] Values of fold reduction in enzymatic activity represent the decrease in AP activity on acquisition of the null *phoP105* allele. These were calculated from logarithmic growth phase cultures and rounded to the nearest whole number.

Claims

1. A vaccine comprising a bacterial cell the virulence of which is attenuated by the constitutive expression of a gene under the control of a two-component regulatory system.

2. The vaccine of claim 1, wherein said constitutive expression is the result of a mutation at a component of said two-component regulatory system.

3. The vaccine of claim 1, wherein said bacterial cell comprises a second mutation which attenuates virulence.

4. The vaccine of claim 1, wherein said bacterial cell is a *Salmonella* cell, said two-component regulatory system is the *phoP* regulatory region, and said gene is a *phoP* regulatory region regulated gene.

5. The vaccine of claim 4, wherein said constitutive expression is the result of a mutation.

6. The vaccine of claim 5, wherein said mutation is in the *phoP* regulatory region.

7. The vaccine of claim 6, wherein said mutation is in the *phoP* gene.

8. The vaccine of claim 6, wherein said mutation is in the *phoQ* gene.

9. The vaccine of claim 6, wherein said mutation is a $phoP^c$ mutation.

10. The vaccine of claim 6, wherein said mutation is a non-revertible mutation.

11. The vaccine of claim 4, wherein said constitutive expression is the result of a change at the promoter of said regulated gene.

12. The vaccine of claim 4, wherein said gene is a *prg* gene.

13. A vaccine comprising a *Salmonella* cell which is attenuated by the decreased expression of a *phoP* regulatory region regulated virulence gene.

14. The vaccine of claim 13, wherein said decrease of expression is the result of a mutation.

15. The vaccine of claim 14, wherein said mutation is in the *prgH* gene.

16. The vaccine of claim 14, wherein said mutation is in the *prgA*, *prgB*, *prgC*, or *prgE* genes.

17. The vaccine of claim 4, wherein said gene the is a *pag* gene.

18. The vaccine of claim 17, wherein said *pag* locus is the *pag*C locus.

19. The vaccine of claim 4, further characterized in that said *Salmonella* cell comprises a first mutation which attenuates virulence and a second mutation which attenuates virulence.

20. The vaccine of claim 19, wherein said first mutation is in a *phoP* regulatory region gene.

21. The vaccine of claim 20, wherein said first mutation is in the *phoP* gene.

22. The vaccine of claim 20, wherein said first mutation is in the *phoQ* gene.

23. The vaccine of claim 20, wherein said first mutation is a $phoP^c$ mutation.

24. The vaccine of claim 19, wherein said first mutation is in a *phoP* regulatory region regulated gene.

25. The vaccine of claim 19, wherein said second mutation is a mutation in an *aro*matic amino acid synthetic gene.

26. The vaccine of claim 25, wherein said second mutation is an *aro* mutation.

27. The vaccine of claim 19, wherein said second mutation is in a *phoP* regulatory region regulated gene.

28. The vaccine of claim 23, wherein said second mutation is in a *prg* locus.

29. The vaccine of claim 13, further characterized in that said *Salmonella* cell comprises two mutant genes, a first mutant gene which attenuates virulence and a second mutant gene which attenuates virulence.

30. The vaccine of claim 29, wherein said second gene is in a *prg* locus.

31. The vaccine of claim 30, wherein said gene is *prgH*.

32. The vaccine of claim 30, wherein said gene is *prgA*, *prgB*, *prgC*, or *prgE*.

33. The vaccine of claim 27, wherein said second mutation is in a *pag* locus.

34. The vaccine of claim 27, wherein said second mutation is a *pag*C mutation.

35. The vaccine of claim 4, wherein said *Salmonella* is of the species *S. typhi*.

36. The vaccine of claim 4, wherein said *Salmonella* is of the species *S. enteriditis* and of the strain *typhimurium*.

37. The vaccine of claim 4, wherein said *Salmonella* is of the species *S. cholerae-suis*.

38. The vaccine of claim 4, wherein said vaccine is a live vaccine.

39. A vaccine comprising a bacterial cell the virulence of which is attenuated by a mutation in a gene under the control of a two-component regulatory system.

40. The vaccine of claim 39, further characterized in that said bacterial cell comprises a virulence attenuating mutation in a second gene.

41. The vaccine of claim 39, wherein said bacterial cell is *Salmonella* cell and said two-component regulatory system is the phoP regulatory region.

42. The vaccine of claim 41, wherein said gene is a *prg* gene.

43. The vaccine of claim 41, wherein said gene is *prgH*.

44. The vaccine of claim 41, wherein said gene is *prgA*, *prgB*, *prgC*, or *prgE*.

45. The vaccine of claim 41, wherein said gene is a *pag* gene.

46. The vaccine of claim 45, wherein said gene is *pagC*.

47. The vaccine of claim 41, wherein said bacterial cell further comprises a mutation in a second gene, said mutation attenuating the virulence of said bacterial cell.

48. The vaccine of claim 47, wherein said second gene is an *aro*matic amino acid biosynthetic gene.

49. The vaccine of claim 48, wherein said second gene is an *aro* gene.

50. A vaccine comprising a *Salmonella* cell comprising a first virulence attenuating mutation in an *aro*matic amino acid biosynthetic gene and a second virulence attenuating mutation in a *phoP* regulatory region gene.

51. The vaccine of claim 50, wherein said first mutation is at an *aro* gene.

52. The vaccine of claim 51, wherein said second mutation is a phoP$^-$ mutation.

53. A bacterial cell which constitutively expresses a gene under the control of a two-component regulatory system and which comprises a virulence attenuating mutation which does not result in constitutive expression of a gene under the control of said two-component regulatory system.

54. The bacterial cell of claim 53, further comprising a mutation in a component of said two-component regulatory system.

55. The bacterial cell of claim 53, wherein said cell is a *Salmonella* cell which expresses a *phoP* regulatory region regulated gene constitutively and which comprises a virulence attenuating mutation which does not result in the constitutive expression of a gene under the control of the *phoP* regulatory region.

56. The bacterial cell of claim 55, wherein said constitutive expression is caused by a mutation in the *phoP* regulatory region.

57. The bacterial cell of claim 55, wherein said constitutive expression is caused by a mutation in the *phoP* gene.

58. The bacterial cell of claim 55, wherein said constitutive expression is caused by a mutation in the *phoQ* gene.

59. The bacterial cell of claim 56, wherein said mutation is a *phoP*^{c} mutation.

60. The bacterial cell of claim 56, wherein said mutation is a deletion.

61. The bacterial cell of claim 55, further characterized in that said virulence attenuating mutation is a in an *aro*matic amino acid synthetic gene.

62. The bacterial cell of claim 61, wherein said virulence attenuating mutation is an *aro* mutation.

63. The bacterial cell of claim 55, wherein said virulence attenuating mutation is in a *phoP* regulatory region gene.

64. The bacterial cell of claim 63, wherein said virulence attenuating mutation is the *phoP* gene.

65. The bacterial cell of claim 63, wherein said virulence attenuating mutation is in the *phoQ* gene.

66. The bacterial cell of claim 55, wherein said virulence attenuating mutation is in a *prg* locus.

67. The bacterial cell of claim 66, wherein said virulence attenuating mutation is in the *prgH* gene.

68. The bacterial cell claim 66, wherein said virulence attenuating mutation is in the *prgA*, *prgB*, *prgC*, or *prgE* gene.

69. The bacterial cell of claim 55, wherein said virulence attenuating mutation is in a *pag* locus.

70. The bacterial cell of claim 55, wherein said virulence attenuating mutation is a *pagC* mutation.

71. The bacterial cell of claim 55, wherein said cell is of the species *S. typhi*.

72. The bacterial cell of claim 55, wherein said cell is of the species *S. enteriditis* and of the strain *typhi*murium.

73. The bacterial cell of claim 55, wherein said *Salmonella* cell is of the species *S. cholerae-suis*.

74. A bacterial cell comprising a virulence attenuating mutation in a gene regulated by a *phoP* regulatory region.

75. The bacterial cell of claim 74, wherein said bacterial cell is a *Salmonella* cell and said virulence attenuating mutation is in a *phoP* regulatory region regulated gene.

76. The bacterial cell of claim 75, wherein said gene is a *prg* gene.

77. The bacterial cell of claim 76, wherein said gene is the *prgH* gene.

78. The bacterial cell of claim 76, wherein said gene is the *prgA*, *prgB*, *prgC*, or *prgE* gene.

79. The bacterial cell of claim 75, wherein said gene is a *pag* gene.

80. The bacterial cell of claim 79, wherein said gene is *pagC*.

81. The bacterial cell of claim 74, further comprising a second mutation which attenuates virulence but which does not result in constitutive expression of a *phoP* regulatory region regulated gene.

82. The bacterial cell of claim 81, wherein said second mutation is in an *aro*matic amino acid synthetic gene.

83. The bacterial cell of claim 82, wherein said second mutation is an *aro* mutation.

84. The bacterial cell of claim 81, wherein said second mutation is in a *phoP* regulatory region gene.

85. The bacterial cell of claim 84, wherein said second mutation is in the *phoP* locus.

86. The bacterial cell of claim 84, wherein said second mutation is in the *phoQ* locus.

87. The bacterial cell of claim 81, wherein said second mutation is in a *phoP* regulating region regulated gene.

88. The bacterial cell of claim 87, wherein said second mutation is in a *pag* locus.

89. The bacterial cell of claim 75, wherein said cell is of the species *S. typhi*.

90. The bacterial cell of claim 75, wherein said cell is of the species *S. enteriditis* and of the strain *typhimurium*.

91. The bacterial cell of claim 75, wherein said cell is of the species *S. cholerae-suis*.

92. A live *Salmonella* cell in which there is inserted into a virulence gene a gene encoding a heterologous protein, or a regulatory element, of said heterologous protein gene.

93. The live *Salmonella* cell of claim 92, wherein said virulence gene is in the *phoP* regulatory region.

94. The live *Salmonella* cell of claim 92, wherein said virulence gene is a phoP regulatory region regulated gene.

95. The live *Salmonella* cell of claim 94, wherein said virulence gene is a *prg* gene.

96. The live *Salmonella* cell of claim 95, wherein said virulence gene is the *prgH* gene.

97. The live *Salmonella* cell of claim 95, wherein said virulence gene is the *prgA*, *prgB*, *prgC*, or *prgE* gene.

98. The live *Salmonella* cell of claim 94, wherein said virulence gene is a *pag* gene.

99. The live *Salmonella* cell of claim 98, wherein said *pag* gene is *pag*C.

100. The live *Salmonella* cell of claim 92, wherein said *Salmonella* cell carries a second mutation that attenuates virulence.

101. The live *Salmonella* cell of claim 100, wherein said second mutation is an *aro* mutation.

102. The live *Salmonella* cell of claim 92, wherein said DNA encoding a heterologous protein is under the control of an environmentally regulated promoter.

103. The live *Salmonella* cell of claim 92, wherein said *Salmonella* cell is of the species *S. typhi*.

104. The live *Salmonella* cell of claim 92, further comprising a DNA sequence encoding T7 polymerase under the control of an environmentally regulated promoter and a T7 transcriptionally sensitive promoter, said T7 transcriptionally sensitive promoter controlling the expression of said heterologous antigen.

105. A vector capable of integrating into the chromosome of *Salmonella* comprising

a first DNA sequence encoding a heterologous protein,

a second DNA sequence encoding a marker, and

a third DNA sequence encoding a *pho*P regulatory region regulated gene product necessary for virulence, said third DNA sequence being mutationally inactivated.

106. The vector of claim 105, wherein said *pho*P regulatory region regulated gene is a *prg* locus.

107. The vector of claim 106, wherein said gene is *prgH*.

108. The vector of claim 106, wherin said gene is *prgA*, *prgB*, *prgC*, or *prgE*.

109. The vector of claim 105, wherein said *phoP* regulatory region regulated gene is a *pag* locus.

110. The vector of claim 109, wherein said *pag* locus is *pagC*.

111. The vector of claim 105, wherein said first DNA sequence is disposed on said vector so as to mutationally inactivate said third DNA sequence.

112. The vector of claim 105, wherein said vector cannot replicate in a wild-type *Salmonella* strain.

113. The vector of claim 105, wherein said first DNA sequence encoding a heterologous protein is under the control of an environmentally regulated promoter.

114. The vector of claim 105, further comprising a DNA sequence encoding T7 polymerase under the control of an environmentally regulated promoter and a T7 transcriptionally sensitive promoter, said T7 transcriptionally sensitive promoter controlling the expression of said first DNA sequence encoding a heterologous protein.

115. A method of vaccinating an animal against a disease caused by a bacterium comprising administering the vaccine of claim 1.

116. The method of claim 115, wherein said bacterium is *Salmonella* and said vaccine is the vaccine of claim 4.

117. A method of vaccinating an animal against a disease caused by a bacterium comprising administering the vaccine of claim 39.

118. The method of claim 115, wherein said bacterium is *Salmonella* and said vaccine is the vaccine of claim 41.

119. A method of vaccinating an animal against a disease caused by *Salmonella* comprising administering the vaccine of claim 50.

120. A vector comprising DNA which encodes the *pagC* gene product.

121. A cell comprising the vector of claim 120.

122. A method of producing the *pagC* gene product comprising culturing the cell of claim 121 and purifying the *pagC* gene product from said cell or culture medium.

123. A purified preparation of the *pagC* gene product.

124. A method of detecting the presence of *Salmonella* in a sample comprising contacting said sample with *pagC* encoding DNA and detecting the hybridization of said *pagC* encoding DNA to nucleic acid in said sample.

125. A vector comprising DNA which encodes the *prgH* gene product.

126. A cell comprising the vector of claim 125.

127. A method of producing the *prgH* gene product comprising culturing the cell of claim 126 and purifying the *prgH* gene product from said cell or culture medium.

128. A purified preparation of the *prgH* gene product.

129. A method of detecting the presence of *Salmonella* in a sample comprising contacting said sample with *prgH* encoding DNA and detecting the hybridization of said *prgH* encoding DNA to nucleic acid in said sample.

130. A method of attenuating the virulence of a bacterium, said bacterium comprising a two-component regulatory system, comprising causing a gene under the control of said two-component system to be expressed constitutively.

131. The method of claim 124, wherein said bacterium is *Salmonella* and said two-component system is the *phoP* regulatory region.

132. A bacterial cell the virulence of which is attenuated by a first mutation in a PhoP regulon and a second mutation in an aromatic amino acid synthetic gene.

133. The bacterial cell of claim 132, wherein said bacterial cell is a *Salmonella* cell.

134. The *Salmonella* cell of claim 133, wherein said *Salmonella* cell is a *Salmonella typhimurium* cell.

135. The *Salmonella* cell of claim 133, wherein said *Salmonella* cell is a *Salmonella enteriditis*.

136. The *Salmonella* cell of claim 135, wherein said *Salmonella* cell is a *Salmonella pylorum* cell.

137. The *Salmonella* cell of claim 135, wherein said *Salmonella* cell is a *Salmonella paratyphi A* cell.

138. The *Salmonella* cell of claim 135, wherein said *Salmonella* cell is a *Salmonella paratyphi B* cell.

139. The *Salmonella* cell of claim 133, wherein said *Salmonella* cell is a *Salmonella cholerasuis* cell.

140. The *Salmonella* cell of claim 133, wherein said *Salmonella* cell is a *Salmonella typhi* cell.

141. The bacterial cell of claim 133, wherein said first mutation comprises a non-revertable null mutation in the PhoP/PhoQ locus.

142. The bacterial cell of claim 141, wherein said mutation comprises a deletion of at least 100 nucleotides.

143. The bacterial cell of claim 142, wherein said mutation comprises a deletion of at least 500 nucleotides.

144. The bacterial cell of claim 143, wherein said mutation comprises a deletion of at least 750 nucleotides.

145. The bacterial cell of claim 144, wherein said mutation comprises a deletion of nucleotides 376 to 1322 of said *PhoP/PhoQ* locus.

146. The bacterial cell of claim 141, wherein said second mutation comprises a non-revertable null mutation in an AroA locus.

147. The bacterial cell of claim 141, wherein said second mutation comprises a non-revertable null mutation in an AroC/AroD locus.

148. The bacterial cell of claim 146, further comprising a mutation in a non-aromatic amino acid synthetic gene, wherein said mutation renders said cell auxotrophic for said non-aromatic amino acid.

149. The bacterial cell of claim 148, wherein said amino acid is histidine.

150. The bacterial cell of claim 149, wherein said *S. typhi* has the genotype $AroA^-$, His^-, $PhoP/PhoQ^-$.

151. The bacterial cell of claim 150, wherein said *S. typhi* is TyH445.

152. The bacterial cell of claim 134, wherein wherein said first mutation comprises a non-revertable null mutation in the PhoP/PhoQ locus.

153. The bacterial cell of claim 152, wherein said mutation comprises a deletion of nucleotides 376-1322 of said *PhoP/PhoQ* locus.

154. The bacterial cell of claim 152, wherein said second mutation comprises a non-revertible null mutation in an AroA locus.

155. The bacterial cell of claim 154, further comprising a mutation in a non-aromatic amino acid synthetic gene, wherein said mutation renders said cell auxotrophic for said non-aromatic amino acid.

156. A vaccine comprising the bacterial cell of claim 132.

157. A substantially pure DNA comprising a sequence encoding *pagD*.

158. The DNA of claim 157, wherein said sequence comprises nucleotides 91 to 354 of SEQ ID NO:5.

159. The DNA of claim 158, further comprising nucleotides 4 to 814 of SEQ ID NO:15.

160. A substantially pure DNA comprising nucleotides 4 to 814 of SEQ ID NO:15.

161. The DNA of claim 160, wherein said DNA sequence comprises nucleotides 562 to 814 of SEQ ID NO:15.

162. The DNA of claim 160, wherein said DNA sequence comprises nucleotides 4 to 776 of SEQ ID NO:15.

163. The DNA of claim 158 and degenerate variants thereof, wherein said sequence encodes a product which comprises essentially the amino acid sequence given in SEQ ID NO:6.

164. A substantially pure DNA comprising a sequence encoding *envE*.

165. The DNA of claim 164, wherein said sequence comprises nucleotides 1114 to 1650 of SEQ ID NO:5.

166. The DNA of claim 165 and degenerate variants thereof, wherein said sequence encodes a product which comprises essentially the amino acid sequence given in SEQ ID NO:7.

167. A substantially pure DNA comprising a sequence encoding *msgA*.

168. The DNA of claim 167, wherein said sequence comprises nucleotides 1825 to 2064 of SEQ ID NO:5.

169. The DNA of claim 168, further comprising nucleotides 1510 to 1824 of SEQ ID NO:5.

170. A substantially pure DNA comprising nucleotides 1510 to 1760 of SEQ ID NO:5.

171. The DNA of claim 168 and degenerate variants thereof, wherein said sequence encodes a product which comprises essentially the amino acid sequence given in SEQ ID NO:8.

172. A substantially pure DNA comprising a sequence encoding *envF*.

173. The DNA of claim 172, wherein said sequence comprises nucleotides 2554 to 3294 of SEQ ID NO:5.

174. The DNA of claim 173, further comprising nucleotides 2304 to 2553 of SEQ ID NO:5.

175. The DNA of claim 173 and degenerate variants thereof, wherein said sequence encodes a product which comprises essentially the amino acid sequence given in SEQ ID NO:9.

176. A substantially pure DNA comprising the sequence given in SEQ ID NO:5 or a fragment thereof.

177. A substantially pure DNA comprising the sequence given in SEQ ID NO:10 or a fragment thereof.

178. A substantially pure DNA comprising a sequence encoding *prgH*.

179. The DNA of claim 178, wherein said sequence comprises nucleotides 688 to 1866 of SEQ ID NO:10.

180. The DNA of claim 179, further comprising nucleotides 1 to 689 of SEQ ID NO:10.

181. The DNA of claim 179 and degenerate variants thereof, wherein said sequence encodes a product which comprises essentially the amino acid sequence given in SEQ ID NO:11.

182. A substantially pure DNA comprising a sequence encoding *prgI*.

183. The DNA of claim 182, wherein said sequence comprises nucleotides 1891 to 2133 of SEQ ID NO:10.

184. The DNA of claim 183, further comprising nucleotides 1 to 689 of SEQ ID NO:10.

185. The DNA of claim 183 and degenerate variants thereof, wherein said sequence encodes a product which comprises essentially the amino acid sequence given in SEQ ID NO:12.

186. A substantially pure DNA comprising a sequence encoding *prgJ*.

187. The DNA of claim 186, wherein said sequence comprises nucleotides 2152 to 2457 of SEQ ID NO:10.

188. The DNA of claim 187, further comprising nucleotides 1 to 689 of SEQ ID NO:10.

189. The DNA of claim 187 and degenerate variants thereof, wherein said sequence encodes a product which comprises essentially the amino acid sequence given in SEQ ID NO:13.

190. A substantially pure DNA comprising a sequence encoding *prgK*.

191. The DNA of claim 190, wherein said sequence comprises nucleotides 2456 to 3212 of SEQ ID NO:10.

192. The DNA of claim 191, further comprising nucleotides 1 to 689 of SEQ ID NO:10.

193. The DNA of claim 191 and degenerate variants thereof, wherein said sequence encodes a product which comprises essentially the amino acid sequence given in SEQ ID NO:14.

194. A bacterial cell, the virulence of which is attenuated by a mutation in one or more genes selected from the group consisting of *pagD*, *pagE*, *pagF*, *pagG*, *pagH*, *pagI*, *pagJ*, *pagK*, *pagL*, *pagM*, *pagN*, *pagP*, *envE*, and *envF*.

195. A bacterial cell, the virulence of which is attenuated by a mutation in one or more genes selected from the group consisting of*pagC*, *pagD*, *pagJ*, *pagK*, *pagM*, and *msgA*.

196. A bacterial cell, the virulence of which is attenuated by a mutation in one or more genes selected from the group consisting of *prgH*, *prgI*, *prgJ*, and *prgK*.

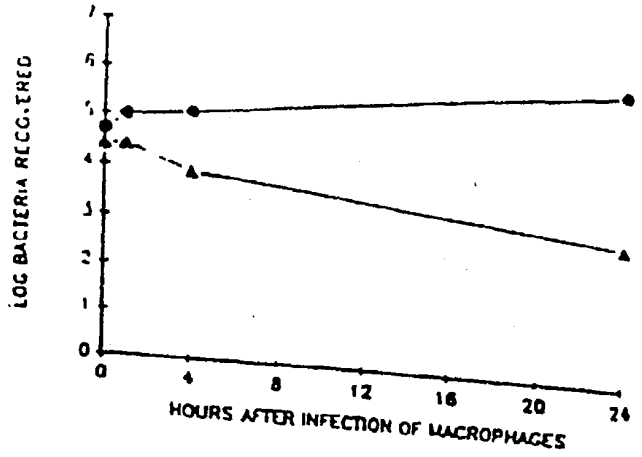
LOG BACTERIA RECOVERED
0
4
8
12
16
20
24
HOURS AFTER INFECTION OF MACROPHAGES

FIGURE 1

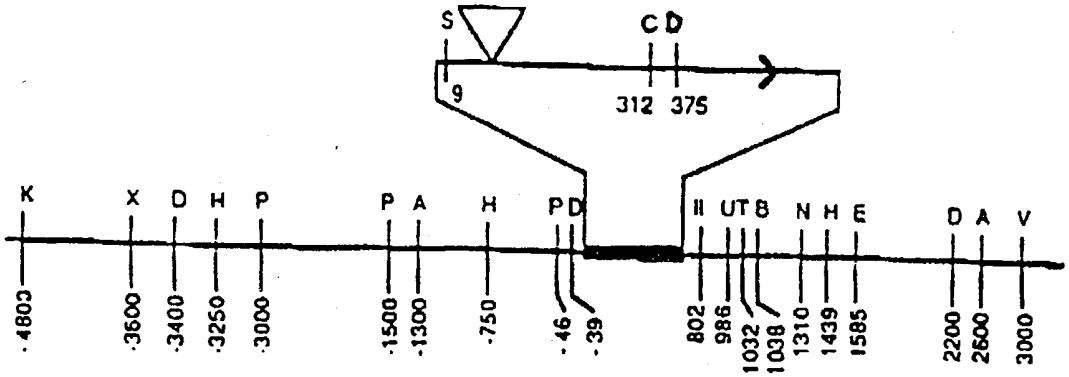
S
C D
9
312 375
K X D H P P A H P D II UT B N H E D A V
-4803 -3600 -3400 -3250 -3000 -1500 -1300 -750 -46 -39 802 986 1032 1038 1310 1439 1585 2200 2800 3000

FIGURE 2

```
        10         20         30         40         50         60         70
GTTAACCACT CTTAATAATA ATGGGTTTTA TAGCGAAATA CACTTTTTTA TCGCGTGTTC AATATTTGCG

        80         90        100        110        120        130        140
TTAGTTATTA TTTTTTTGGA ATGTAAATTC TCTCTAAACA CAGGTGATAT TTATGTTGGA ATTGTGGTGT

       150        160        170        180        190        200        210
TGATTCTATT CTTATAATAT AACAACAAAT GTTGTAACTG ATAGATATAT TAAAAGATTA AATCCGAGCG
                                  *--------->
       220        230        240        250        260        270        280
GGAATAAAGC GTGCTAAGCA TCATCGTGAA TATGATTACA GCGCCTGCGA TGGCATATAA CCGTATTGCG

       290        300        310        320        330        340        350
GATGGAGCGT CACGTGAGGA CTGTGAAGCA CAATGCGATA TGTTCTGATT ATATGGCGAG TTTGCTTAAT

       360        370        380        390        400        410        420
CACATGTTTT TAGCCGAACG GTGTCAAGTT TCTTAATGTG GTTGTGAGAT TTTCTCTTTA AATATCAAAA

       430        440        450        460        470        480        490
TGTTCCATGG GTGATTTGTT GTTCTATAGT GGCTAAACAC TTTATGGTTT CTGTTAAATA TATATGCGTC

       500        510        520        530        540        550        560
ACAAAAATTA GCATTCAAAT CTATAAAAGT TAGATGACAT TGTAGAACCC GTTACCTAAA TGAGCGATAG

       570        580        590        600        610        620        630
AGTCCTTCGG TAGTAAAAAT ATGTTTCAGG AAGTAAACAC ATCAGCAGCG ATACCGCTGA ATTATTCGTG

       640        650        660        670        680        690        700
GTTTTGTCGA TTCCGCATAG TGCGGATAAC TGAATGCCGG ATCGGTACTG CAGGTGTTTA AACACACCGT

       710        720    728
AAATAATAAG TAGTATTAAG GAGTTGTT

ATG AAA AAT ATT ATT TTA TCC ACT TTA GTT ATT ACT ACA AGC GTT TTG GTT GTA  782
MET LYS ASN ILE ILE LEU SER THR LEU VAL ILE THR THR SER VAL LEU VAL VAL   18

AAT GTT GCA CAG GCC GAT ACT AAC GCC TTT TCC GTG GGG TAT GCA CGG TAT GCA  836
ASN VAL ALA GLN ALA ASP THR ASN ALA PHE SER VAL GLY TYR ALA ARG TYR ALA   36

CAA AGT AAA GTT CAG GAT TTC AAA AAT ATC CGA GGG GTA AAT GTG AAA TAC CGT  890
GLN SER LYS VAL GLN ASP PHE LYS ASN ILE ARG GLY VAL ASN VAL LYS TYR ARG   54

TAT GAG GAT GAC TCT CCG GTA AGT TTT ATT TCC TCG CTA AGT TAC TTA TAT GGA  944
TYR GLU ASP ASP SER PRO VAL SER PHE ILE SER SER LEU SER TYR LEU TYR GLY   72

GAC AGA CAG GCT TCC GGG TCT GTT GAG CCT GAA GGT ATT CAT TAC CAT GAC AAG  998
ASP ARG GLN ALA SER GLY SER VAL GLU PRO GLU GLY ILE HIS TYR HIS ASP LYS   90

TTT GAG GTG AAG TAC GGT TCT TTA ATG GTT GGG CCA GCC TAT CGA TTG TCT GAC 1052
PHE GLU VAL LYS TYR GLY SER LEU MET VAL GLY PRO ALA TYR ARG LEU SER ASP  108

AAT TTT TCG TTA TAC GCG CTC GCG GGT GTC GGC ACG GTA AAG GCG ACA TTT AAA 1106
ASN PHE SER LEU TYR ALA LEU ALA GLY VAL GLY THR VAL LYS ALA THR PHE LYS  126

GAA CAT TCC ACT CAG GAT GGC GAT TCT TTT TCT AAC AAA ATT TCC TCA AGG AAA 1160
GLU HIS SER THR GLN ASP GLY ASP SER PHE SER ASN LYS ILE SER SER ARG LYS  144

ACG GGA TTT GCC TGG GGC GCG GGT GTA CAG ATG AAT CCG CTG GAG AAT ATC GTC 1214
THR GLY PHE ALA TRP GLY ALA GLY VAL GLN MET ASN PRO LEU GLU ASN ILE VAL  162
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FIGURE 3 page 1 of 2

GTC GAT GTT GGG [illegible] GAA GGA AGC AAC ATC TCC TCT AC [illegible]AA ATA AAC GGC TTC 1268
VAL ASP VAL GLY [illegible] GLU GLY SER ASN ILE SER SER TH[illegible] LYS ILE ASN GLY PHE 180

AAC GTC GGG GTT GCA TAC CGT TTC TGA AAAGC --- 1300
ASN VAL GLY VAL GLY TYR ARG PHE - 188

1310 1320 1330 1340 1350 1360 1370
ATAAGGTATG CGGAAGGTTC GCGTTCCGGA CCGCCAGTCA ATAAAACAGG GCTTCTTTAC CAGTGACACG

1380 1390 1400 1410 1420 1430 1440
TACGTGCCTG TCTTTTCTCT CTTCGTCATA CTCTCTTCGT CATAGTGACG CTGTACATAA CATCTCACTA

1450 1460 1470 1480 1490 1500 1510
GCATAAGCAC AGATAAAGGA TTGTGGTAAG CAATCAAGGT TGCTCAGGTA GGTGATAAGC AGGAAGGAAA

1520 1530 1540 1550 1560 1570 1580
ATCTGGTGTA AATAACGCCA GATCTCACAA GATTCAGTCT GAAAAATTTT CCTGGAATTA ATCACAATGT

1590 1600 1610 1620 1630 1640 1650
CATCAAGATT TTGTCACCGC CTTCGCATAT TGTACCTGCCG CTGAACGAC TACTGAAAAG TACCAAGGTA

1660 1670 1680 1690 1700 1710 1720
TGTATTTTAT CCAGGAGAGC ACCTTTTTTG CGCCTGGCAG AAGTCCCCAG CCGCCACTAC CTCAGCTCGA

1730 1740 1750 1760 1770 1780 1790
TAGAGCATCA ACCTCCTAA GTTGATGGTGC GAGGTTCGAG GCCTCGGTGG CGGTCCAATG TGGTTATCGT

1800 1810 1820 1830 1840 1850 1860
ATAATGTTAT TACGTCAGT GTCAGGCTGAT GATGTGGGTT CGACTCCCAC TGACCACTTC AGTTTTGAAT

1870 1880 1890 1900 1910 1920 1930
AAGTATTGTC TCGCAACCC TGTTACAGAAT AATTTCATTT ATTACGTGAC AAGATAGTCA TTTATAAAAA

1940 1950 1960 1970 1980 1990 2000
ATGCACAAAA ATGTTATTG TCTTTTATTAC TTGTGAGTTG TAGATTTTTC TTATGCGGTG AATCCCCCTT

2010 2020 2030 2040 2050 2060 2070
TGCGGCGGGG CGTCCAGTC AAATAGTTAAT GTTCCTCGCG AACCATATTG ACTGTGGTAT CGTTCACCGG

2080 2090 2100 2110 2120 2130 2140
GAGGCACCCG GCACCGCAA TTTTTTATAAA ATGAAATTCA CACCCTATGG TTCAGAGCGG TGTCTTTTTA

2150 2160 2170 2180 2190 2200 2210
CATCAGGTGG GCAAGCATA ATGCAGGTTAA CTTGAAAGAT ACGATCAATA GCAGAAACCA GTGATTTCGT

2220 2230 2240 2250 2260 2270 2280
TTATGGCCTG GGGATTTAA CCGCGCCAGAG CGTATGCAAG ACCCTGGCGC GGTTGGCCGG TGATCGTTCA

2290 2300 2310
ATAGTGCGAA TATGAATGG TTACCAGCCGC TGCGAATTC (Sequence I.D. No. 1)

FIGURE 3 page 2 of 2

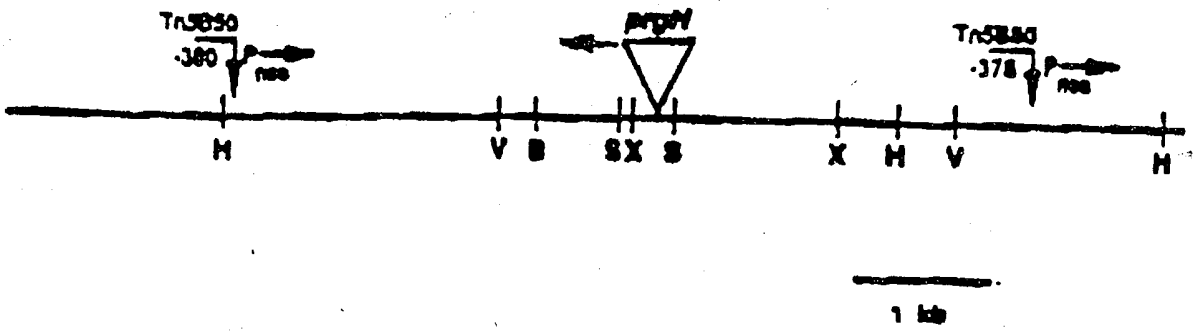

FIGURE 4

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5'      GAG CGC ATT ATC AGA TAA ATT GAT TTA TTTCTCACT
TTC     ATT CTA TTT TCA   TCA
GGA     ATC CCT GTG TCC TGT GCG GTA ATC TGC TGCTATCGA
GAA     CGA CAG ACA TCG
CTA     ACA GTA TAT ATG GAA ACA TCA AAA GAG AAGACGATA
ACA     AGC CCA GGG CCA TAC
ATA     GTT CGA TTA CTT AAC AGC TCA CTG AAC GGCTGTGAG
TTT     CCA TTG CTG ACA GGC
CGA     ACA CTC TTT GTG GTA GGT CAG AGT GAT GCGCTCACT
GCT     TCA GGT CAA CTC CCT
GAT     ATA CCT GCC GAT AGC TTT TTT ATC CCG CTGGACCAT
GGC     GGA GTA AAT TTT GAA
ATC     CAG GTG GAT ACG GAT GCG ACC GAA ATT ATACTCCAT
GAG     CTG AAA GAA GGA AAT
TCT     GAA TCT CGT TCG GTG CAA TTA AAT ACG CCAATACAG
GTC     GGT GAA TTG CTT ATC
CTG     ATT CGC CCG GAA AGC GAG CCG TGG GTG CCCGAGCAG
CCT     GAG AAG TTA GAA ACG
TCT     GCA AAA AAG AAC GAG CCG CGT TTT AAA AACGGAATT
GTA     GCA GCA CTG GCC
```

FIGURE 5 page 1 of 2

```
GGG     TTT TTT ATA TTG GGA ATT GGG ACT GTG GGGACGTTA
TGG     ATA CTT AAC TCG CCG
CAG     CGG CAG GCC CGA GAG CTC GAT TCG TTA TTGGGGCAG
GAG     AAG GAG CGT TTT CAG  GTG     TTG CCA GGCC 3'
```

FIGURE 5 page 2 of 2

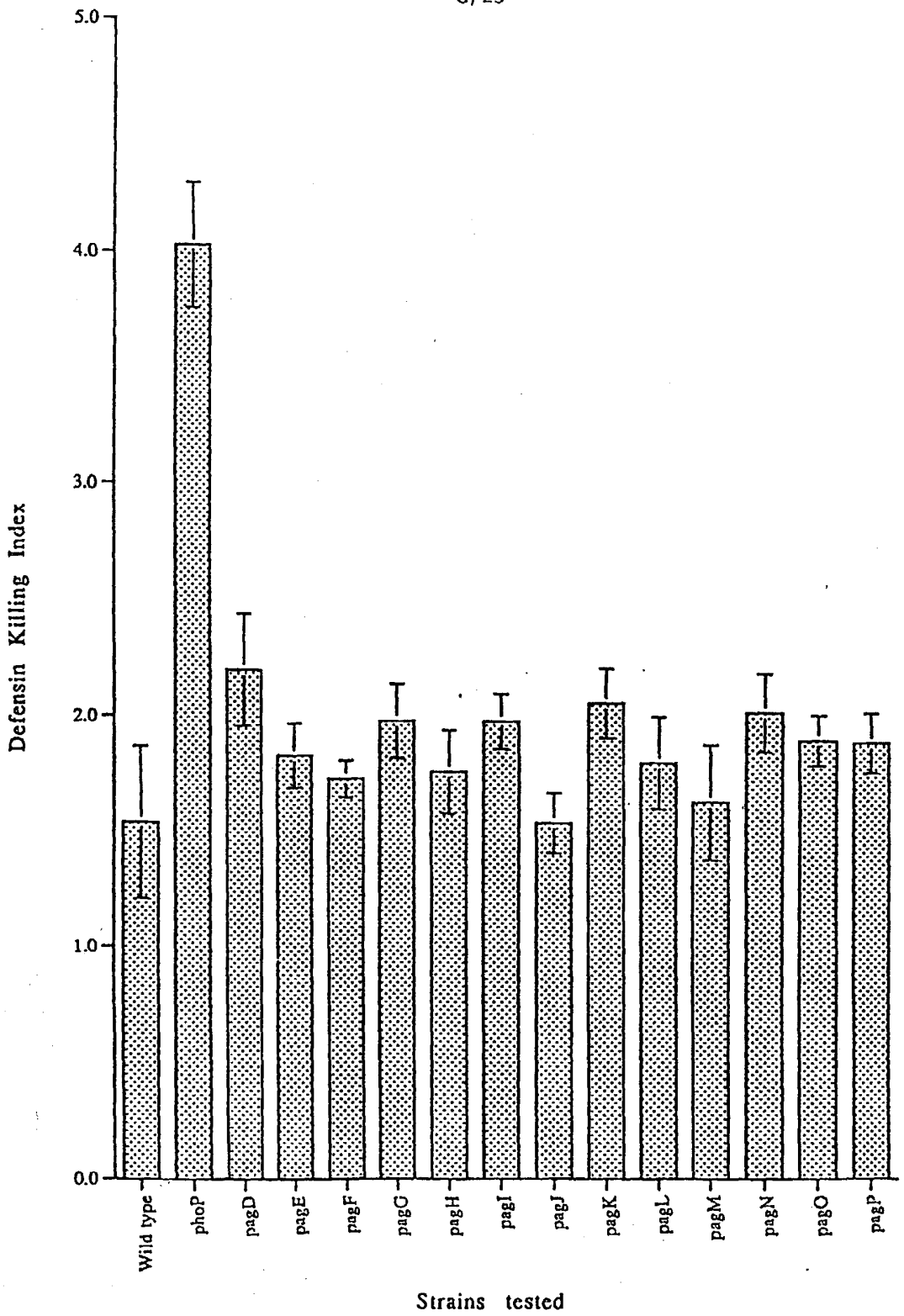

Defensin Killing Index
5.0
4.0
3.0
2.0
1.0
0.0
Wild type
phoP
pagD
pagE
pagF
pagG
pagH
pagI
pagJ
pagK
pagL
pagM
pagN
pagO
pagP
Strains tested

FIGURE 6

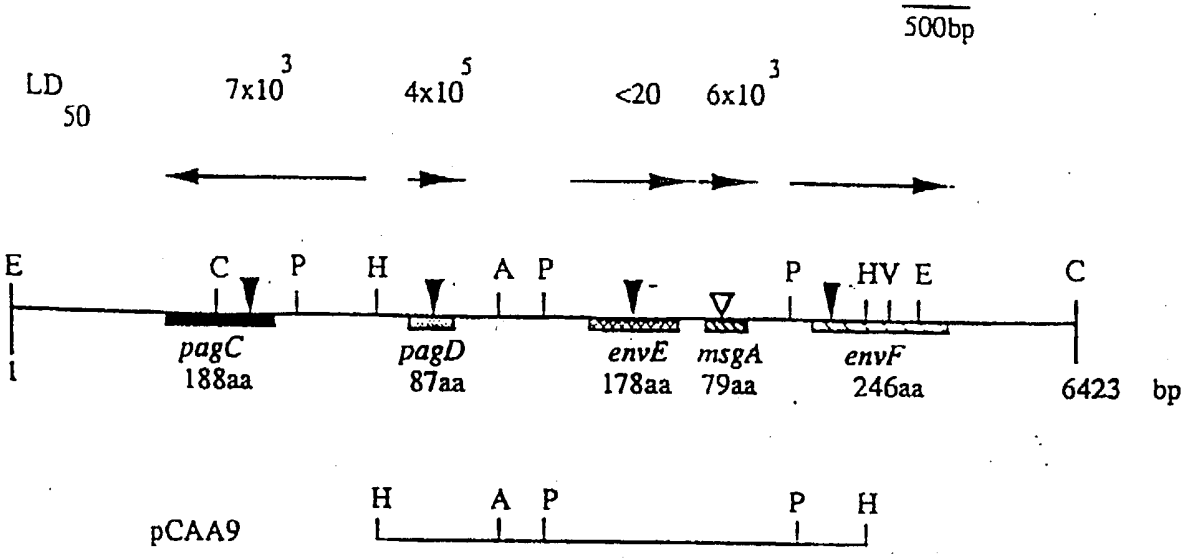

500bp
LD 50
7x10^3
4x10^5
<20
6x10^3
E
C
P
H
A
P
P
HV
E
C
pagC
188aa
pagD
87aa
envE
178aa
msgA
79aa
envF
246aa
1
6423 bp
pCAA9
H
A
P
P
H

FIGURE 7

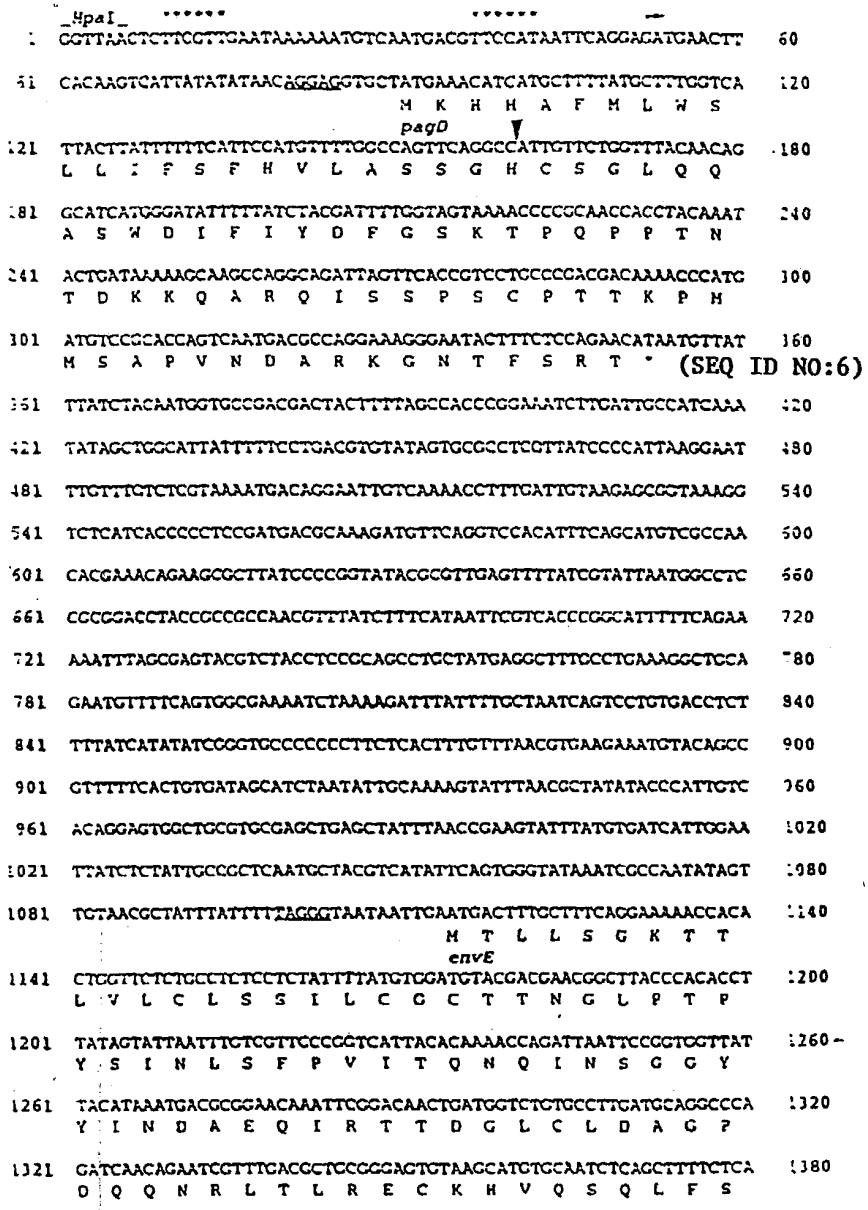


FIGURE 8 page 1 of 3

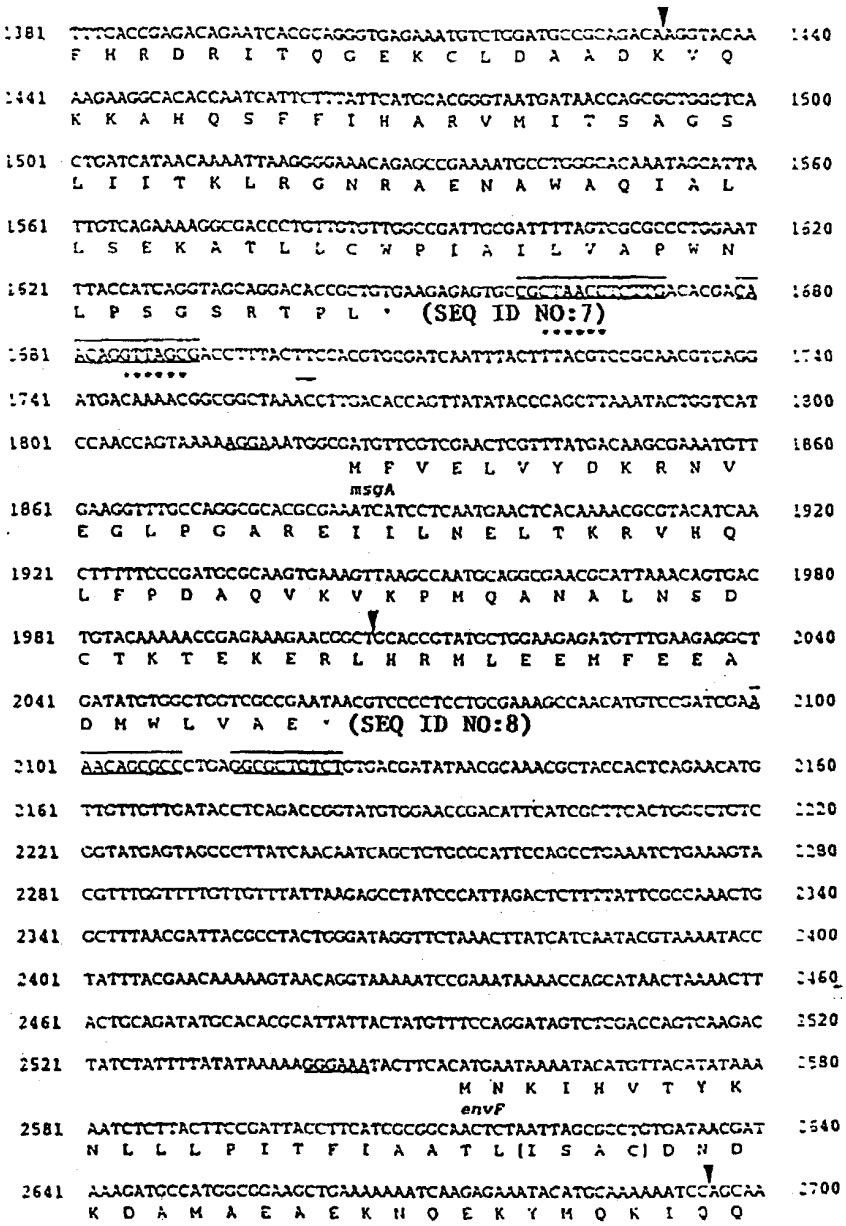


FIGURE 8 page 2 of 3

```
2701 AAAGAGCACCAGCAATCAATGTTCTTTTACGACAAAGCCGAAATGCAAAAAGCTATTGCC 2760
     K  E  H  Q  Q  S  M  F  F  Y  D  K  A  E  M  Q  K  A  I  A

2761 AATATCAACGCAAAAGGTGGAGCCAATCTTGCGATTATTGAAGTCCGTTTCTTCAAGGGC 2820
     N  I  N  A  K  G  G  A  N  L  A  I  I  E  V  R  F  F  K  G

2821 GGGTATTCATTCATTCGACAAAGCGTTAACACCCCTGCTAAAGTAGAGGTGTTTAAATTT 2880
     G  Y  S  F  I  R  Q  S  V  N  T  P  A  K  V  E  V  F  K  F

2881 AACAACGGCTACTGGGGGGGACCTTCGCCTGTCAATTTAACCATCTTTGGCACTATAACA 2940
     N  N  G  Y  W  G  G  P  S  P  V  N  L  T  I  F  G  T  I  T

2941 GAGGAGCAAAAACAAGAAGCACTAAAAGAGGCTTTATTCAAATTCGACTCGATCAATTTC 3000
     E  E  Q  K  Q  E  A  L  K  E  A  L  F  K  F  D  S  I  N  F

3001 AGCATTATACCAGAGCGTATTCAGGAAACAATTAAACGCGCTAACGCCAGTGGCATCATT 3060
     S  I  I  P  E  R  I  Q  E  T  I  K  R  A  N  A  S  G  I  I

3061 TCCGTTACGGAAGATAGCGATATCGTTGTACGAGCAGAGATAGCTCATAATGGCGAATTC 3120
     S  V  T  E  D  S  D  I  V  V  R  A  E  I  A  H  N  G  E  F

3121 GTCTATGACATTACCATCACTGCTAAAAATACAGCACGTGCGGTAATGACCTTAAATAAG 3180
     V  Y  D  I  T  I  T  A  K  N  T  A  R  A  V  M  T  L  N  K

3181 GATGGTTCTATTGCCCGATATGAGATCAAAGAACCTTTCGCCCCAAAAAAAGAAGCCGAA 3240
     D  G  S  I  A  G  Y  E  I  K  E  P  F  A  P  K  K  E  A  E

3241 AAAGCACAGCAACTTGTTGAACAATCGAGAAAAGACATTGAAAGTCCAGCGTAAAAAAGC 3300
     K  A  Q  Q  L  V  E  Q  S  R  K  D  I  E  S  P  A  *  (SEQ ID NO:9)

3301 AGCTGGAAAGATCAACGAAATACAGCAGACATTTAAAAATAGCAGGCGATACAAACATTC 3360

3361 ATAAAAATTATAGCGCGAAAGAGCGCGTGCCAGGTACTAAGGCACTGCTTGAAGACAGCG 3420

3421 AATCGCTATTTCATTCTCTGACACTGTAATTTTTCGTACTCAAGATGTTTATTTATTGAG 3480

3481 TCTTTTGTGGATAACCAGGTGAAGTTATGTGACGCCAGGAATCTATTCCAGCGGGCGTAC 3540

3541 TTGTTGGAGCCAGTGTGAAGCCGGGCAGCGCGCAGAAACCGGAGCGTATACGTTGTACGT 3600

3601 AAGAATTTCGAGCACTGCCCGACCTAAAAATGATCAATAAAATAGATATTTTAAAGAGGT 3660

3661 AATATGAAGAATTTTTTTCAAAATAATTACTGATTTCATCGCGGATATTTCCCTTGATCTA 3720

3721 TTTGCTATATTTTTATGCATGTTATTCGTATACAAAACAGGACCATCAATTGGTGTGATA 3780

3781 TCATTTTTTATTGCATTAATTATTTATATCATTCTTCATTTTTTTTTACTCATTTCTTGA 3840

3841 AAAAATCATAAAAAAAAATATTCAAATAAGTATTTAAAATTATTGTTTTGTGGTACAAATT 3900

3901 CAGCGCAATAAAACAGAGCAACTAAAAAAAATTAGGCGTAGCGAAGTGGAAAAGGACTGT 3960

3961 CATGTACTGGACCGTGAGCTGGTCGGGAGAGCAATGTACGGGAAAGAGCGAAATACTGTC 4020
                    _ClaI_
4021 ATTGATATGAGCAGGAATATCCAT 4044 (SEQ ID NO:5)
```

FIGURE 8 page 3 of 3

```
TTTTGGTTTGCTGCNCGTTTGGGATAACTGCATAGAGAGCGGCCAAGTCGCTTGCGGTCG
        10                  30                  50

GTATCTCGAGTATATCGAAATCCATGTGGCCATTGACCTCTTCAAGCGCTCACGTTAACT
        70                  90                  110

ACCTGCTCTTTTTTGAGCACCAACATCCCAGGTTCGTCACAGTAAATCGTATCGTGATTA
       130                 150                 170

TTGCTAATCGTCAGTTTACCGCTCCGAAAGCAAACTANAAGTGAAACTGCTTACATAAAG
       190                 210                 230

ATTTTTGATGGTAACCTGCTGAGTCTGACTTTTAATTTGCTGCCGGGTATTTGTCAAAAG
       250                 270                 290

TGATTTTAATTTCTGTAAGTTATCTGCGGCAGGACGCTGATGACTATTACTTACAAAGGT
       310                 330                 350

TACATTTTCCATATTATCCCTTTGTTGAACTTATTTTAATGTTCCTTACTGGTATCCTAC
       370                 390                 410

TGAAAAAATCTGAGTTGTAAATGCTCTTTATTAGCGTGTGTTGGCAATGGTCTGATTGTT
       430                 450                 470

ACACCAAAAGAACCCAAATTTGGGTAATTTATCTACAGTAGTTTAAGCCCCAATGGGGAT
       490                 510                 530

GATGGTTCTTTTAATATGTGTTGAGACGCATTATACAGAATAAATTGATTTTATTTCTCA
       550                 570                 590

CTTTTCATTCTATTTTCATCAGGAATCCCTGTGTCCTGTGCGGTAATCTGCTGCTATCGA
       610                 630                 650
                           prgH
GAACGACAGACATCGCTAACAGTATATATGGAAACATCAAAAGAGAAGACGATAACAAGC
                           M  E  T  S  K  E  K  T  I  T  S
       670                 690                 710
```

FIGURE 9 page 1 of 5

```
CCAGGGCCATACATAGTTCGATTACTTAACAGCTCACTGAACGGCTGTGAGTTTCCATTG
P  G  P  Y  I  V  R  L  L  N  S  S  L  N  G  C  E  F  P  L
      730                 750                 770
CTGACAGGCCGAACACTCTTTGTGGTAGGTCAGAGTGATGCGCTCACTGCTTCAGGTCAA
L  T  G  R  T  L  F  V  V  G  Q  S  D  A  L  T  A  S  G  Q
      790                 810                 830

CTCCCTGATATACCTGCCGATAGCTTTTTTATCCCGCTGGACCATGGCGGAGTAAATTTT
L  P  D  I  P  A  D  S  F  F  I  P  L  D  H  G  G  V  N  F
      850                 870                 890

GAAATCCAGGTGGATACGGATGCGACCGAAATTATACTCCATGAGCTGAAAGAAGGAAAT
E  I  Q  V  D  T  D  A  T  E  I  I  L  H  E  L  K  E  G  N
      910                 930                 950

TCTGAATCTCGTTCGGTGCAATTAAATACGCCAATACAGGTCGGTGAATTGCTTATCCTG
S  E  S  R  S  V  Q  L  N  T  P  I  Q  V  G  E  L  L  I  L
      970                 990                1010

ATTCGCCCGGAAAGCGAGCCGTGGGTGCCCGAGCAGCCTGAGAAGTTAGAAACGTCTGCA
I  R  P  E  S  E  P  W  V  P  E  Q  P  E  K  L  E  T  S  A
     1030                1050                1070

AAAAAGAACGAGCCGCGTTTTAAAAACGGAATTGTAGCAGCACTGGCCGGGTTTTTTATA
K  K  N  E  P  R  F  K  N  G  I  V  A  A  L  A  G  F  F  I
     1090                1110                1130

TTGGGAATTGGGACTGTGGGGACGTTATGGATACTTAACTCGCCGCAGCGGCAGGCCGCA
L  G  I  G  T  V  G  T  L  W  I  L  N  S  P  Q  R  Q  A  A
     1150                1170                1190

GAGCTCGATTCGTTATTGGGGCAGGAGAAGGAGCGTTTTCAGGTGTTGCCAGGCCGGGAC
E  L  D  S  L  L  G  Q  E  K  E  R  F  Q  V  L  P  G  R  D
     1210                1230                1250

AAAATGCTCTATGTCGCTGCGCAAAATGAAAGAGATACGTTGTGGGCTCGTCAGGTTTTA
K  M  L  Y  V  A  A  Q  N  E  R  D  T  L  W  A  R  Q  V  L
     1270                1290                1310

GCGAGGGGCGATTATGATAAAAATGCGCGAGTGATTAACGAAAACGAAGAAAATAAGCGT
A  R  G  D  Y  D  K  N  A  R  V  I  N  E  N  E  E  N  K  R
     1330                1350                1370

ATCTCTATCTGGCTGGATACCTATTATCCGCAGCTGGCTTATTATCGGATTCATTTCGAT
I  S  I  W  L  D  T  Y  Y  P  Q  L  A  Y  Y  R  I  H  F  D
     1390                1410                1430

GAGCCGCGTAAACCCGTTTTCTGGCTAAGCCGCCAGCGAAACACGATGAGCAAGAAAGAG
E  P  R  K  P  V  F  W  L  S  R  Q  R  N  T  M  S  K  K  E
     1450                1470                1490

CTCGAGGTGTTAAGTCAAAAGCTGAGAGCGCTAATGCCTTACGCGGATTCGGTTAACATC
L  E  V  L  S  Q  K  L  R  A  L  M  P  Y  A  D  S  V  N  I
     1510                1530                1550

ACGTTGATGGACGATGTTACCGCAGCAGGCCAGGCGGAAGCGGGGCTAAAACAGCAGGCG
T  L  M  D  D  V  T  A  A  G  Q  A  E  A  G  L  K  Q  Q  A
```

FIGURE 9 page 2 of 5

```
      1570                1590                1610

TTACCTTATTCCCGCAGGAATCATAAGGGGGGCGTAACGTTTGTTATTCAGGGGGCGCTC
L  P  Y  S  R  R  N  H  K  G  G  V  T  F  V  I  Q  G  A  L
      1630                1650                1670

GATGATGTAGAAATACTCAGAGCCCGTCAATTTGTCGATAGCTATTACCGCACATGGGGA
D  D  V  E  I  L  R  A  R  Q  F  V  D  S  Y  Y  R  T  W  G
      1690                1710                1730

GGACGCTATGTGCAGTTTGCGATCGAATTAAAAGATGACTGGCTCAAGGGGCGCTCATTT
G  R  Y  V  Q  F  A  I  E  L  K  D  D  W  L  K  G  R  S  F
      1750                1770                1790

CAGTACGGGGCGGAAGGTTATATCAAAATGAGCCCAGGCCATTGGTATTTCCCAAGCCCA
Q  Y  G  A  E  G  Y  I  K  M  S  P  G  H  W  Y  F  P  S  P
      1810                1830                1850
                                  prgI
CTTTAATTTAACGTAAATAAGGAAGTCATTATGGCAACACCTTGGTCAGGCTATCTGGAT
L  *** (SEQ ID NO: 11)      M  A  T  P  W  S  G  Y  L  D
      1870                1890                1910

GACGTCTCAGCAAAATTTGATACGGGCGTTGATAATCTACAAACGCAGGTAACAGAGGCG
D  V  S  A  K  F  D  T  G  V  D  N  L  Q  T  Q  V  T  E  A
      1930                1950                1970

CTGGATAAATTAGCAGCAAAACCCTCCGATCCGGCGCTACTGGCGGCGTATCAGAGTAAG
L  D  K  L  A  A  K  P  S  D  P  A  L  L  A  A  Y  Q  S  K
      1990                2010                2030

CTCTCGGAATATAACTTGTACCGTAACGCGCAATCGAACACGGTAAAAGTCTTTAAGGAT
L  S  E  Y  N  L  Y  R  N  A  Q  S  N  T  V  K  V  F  K  D
      2050                2070                2090
                                                      prgJ
ATTGATGCTGCCATTATTCAGAACTTCCGTTAATCAGTTATAAGGTGGATTATGTCGATT
I  D  A  A  I  I  Q  N  F  R  * (SEQ ID NO:12)      M  S  I
      2110                2130                2150

GCAACTATTGTCCCTGAGAATGCCGTTATAGGGCAGGCGGTCAATATCAGGTCTATGGAA
A  T  I  V  P  E  N  A  V  I  G  Q  A  V  N  I  R  S  M  E
      2170                2190                2210

ACGGACATTGTCTCGCTGGATGACCGGCTACTCCAGGCTTTTTCTGGTTCGGCGATTGCC
T  D  I  V  S  L  D  D  R  L  L  Q  A  F  S  G  S  A  I  A
      2230                2250                2270

ACGGCTGTGGATAAACAGACGATTACCAACAGGATTGAGGACCCTAATCTGGTGACGGAT
T  A  V  D  K  Q  T  I  T  N  R  I  E  D  P  N  L  V  T  D
      2290                2310                2330

CCTAAAGAGCTGGCTATTTCGCAAGAGATGATTTCAGATTATAACCTGTATGTTTCTATG
P  K  E  L  A  I  S  Q  E  M  I  S  D  Y  N  L  Y  V  S  M
      2350                2370                2390
                                                      prgK
GTCAGTACCCTTACTCGTAAAGGAGTCGGGGCTGTTGAAACGCTATTACGCTCATGATTC
V  S  T  L  T  R  K  G  V  G  A  V  E  T  L  L  R  S  *** (SEQ ID NO:13
      2410                2430                2450   M  I  R
```

FIGURE 9 page 3 of 5

```
GTCGATATCTATATACTTTTCTGCTGGTAATGACCCTTGCCGGCTGTAAGGATAAGGATC
  R  Y  L  Y  T  F  L  L  V  M  T  L  A  G  C  K  D  K  D  L
     2470              2490              2510

TTTTAAAAGGACTGGACCAGGAACAGGCTAATGAGGTCATTGCCGTTCTGCAAATGCACA
  L  K  G  L  D  Q  E  Q  A  N  E  V  I  A  V  L  Q  M  H  N
     2530              2550              2570

ATATAGAGGCGAATAAAATTGATAGCGGAAAATTGGGCTATAGCATTACCGTTGCTGAGC
  I  E  A  N  K  I  D  S  G  K  L  G  Y  S  I  T  V  A  E  P
     2590              2610              2630

CTGATTTTACCGCTGCGGTGTACTGGATTAAAACTTATCAGCTTCCTCCCCGGCCACGGG
  D  F  T  A  A  V  Y  W  I  K  T  Y  Q  L  P  P  R  P  R  V
     2650              2670              2690

TGGAAATAGCGCAGATGTTCCCGGCGGATTCGCTGGTATCGTCTCCGCGAGCTGAAAAGG
  E  I  A  Q  M  F  P  A  D  S  L  V  S  S  P  R  A  E  K  A
     2710              2730              2750

CCAGGTTATATTCGGCTATTGAACAGCGACTGGAACAGTCATTACAGACGATGGAGGGCG
  R  L  Y  S  A  I  E  Q  R  L  E  Q  S  L  Q  T  M  E  G  V
     2770              2790              2810

TGCTCTCCGCCAGGGTCCATATTAGTTATGATATTGATGCTGGTGAAAATGGCCGCCCGC
  L  S  A  R  V  H  I  S  Y  D  I  D  A  G  E  N  G  R  P  P
     2830              2850              2870

CAAAACCTGTTCATCTGTCGGCATTAGCCGTATATGAACGAGGTTCGCCGCTTGCGCATC
  K  P  V  H  L  S  A  L  A  V  Y  E  R  G  S  P  L  A  H  Q
     2890              2910              2930

AGATCAGCGATATCAAGCGTTTCTTAAAGAATAGTTTTGCCGATGTGGATTATGACAACA
  I  S  D  I  K  R  F  L  K  N  S  F  A  D  V  D  Y  D  N  I
     2950              2970              2990

TTTCTGTTGTGTTGTCAGAACGTTCTGATGCCCAATTACAGGCTCCCGGCACACCAGTAA
  S  V  V  L  S  E  R  S  D  A  Q  L  Q  A  P  G  T  P  V  K
     3010              3030              3050

AACGTAATTCTTTTGCAACCAGTTGGATTGTTTTGATTATTTTGTTATCCGTGATGTCAG
  R  N  S  F  A  T  S  W  I  V  L  I  I  L  L  S  V  M  S  A
     3070              3090              3110

CAGGCTTTGGCGTCTGGTATTACAAAAACCATTATGCCCGCAATAAGAAAGGCATAACGG
  G  F  G  V  W  Y  Y  K  N  H  Y  A  R  N  K  K  G  I  T  A
     3130              3150              3170

CTGATGATAAGGCGAAATCGTCAAATGAATAGGCAGCCATTACCCATTATCTGGCAAAGA
  D  D  K  A  K  S  S  N  E  ***  (SEQ ID NO:14)
     3190              3210              3230

TCATTTTTGATCCGTTATCGTATATCCATCCTCAGCGGTTGCAGATAGCGCCGGAAATGA
     3250              3270              3290

TTGTCAGACCGCGCCACGCGAAATGAGTTAATACTGGCGGCATGGCGGCGGCTTAAGAAC
     3310              3330              3350
```

GGAGAAAAGGAGTGTATTCAAAACTCACTGACGCAGCTGTGGCTGCTCAGTGGCGCCGAC--
3370 3390 3410

TGCCGCAAGTAGCGTATTTACTAAACTGAGAGCCGATCTGGCAAGGCAGGGAGCCTTGCT
3430 3450 3470

TGGCCTAGCCGGATTGGGCGAAATGAGTTAATACTGGCGGCATGGCGGCTTGCCAT (SEQ ID NO:1(
3490 3510 3530

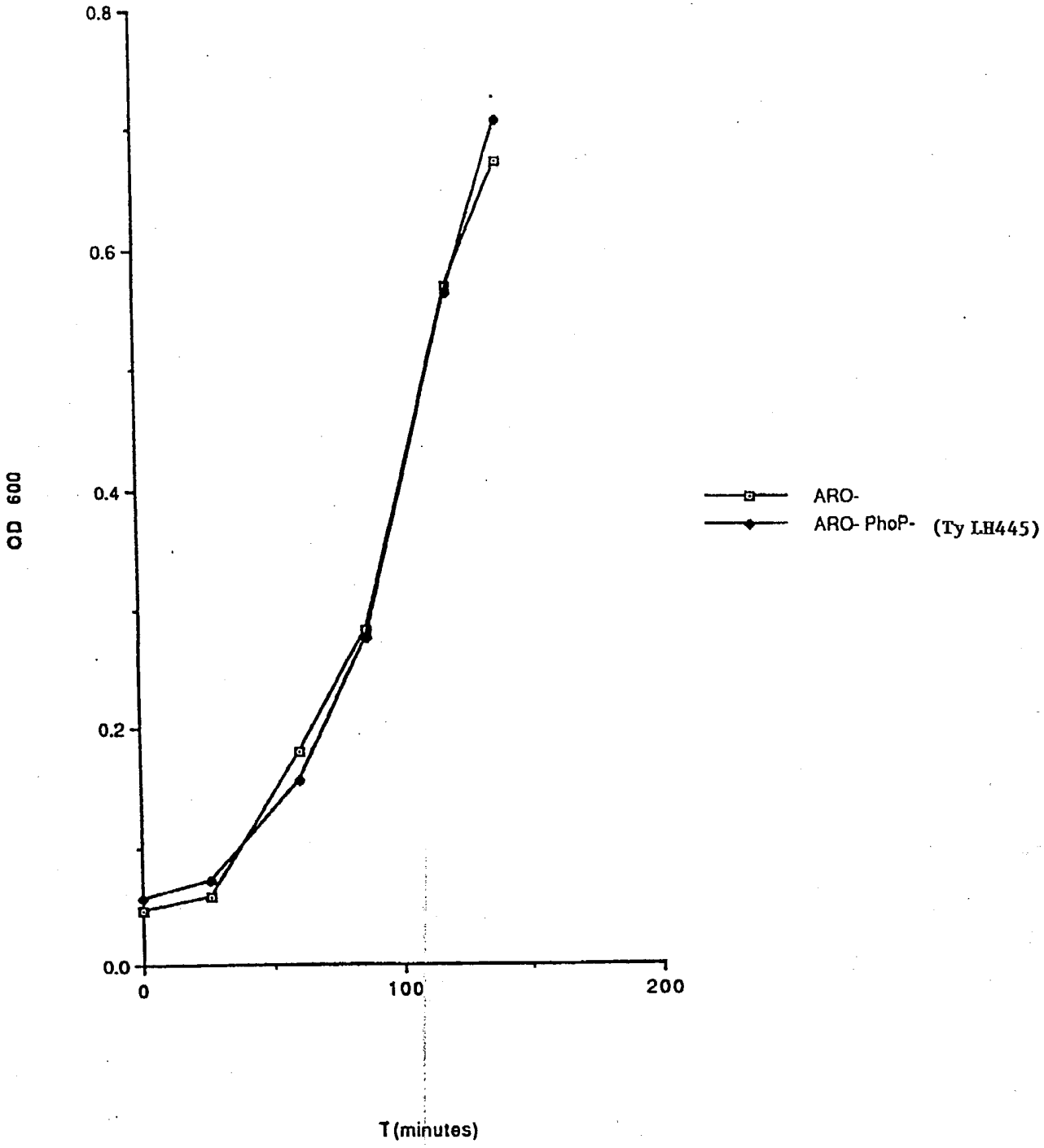


FIGURE 10

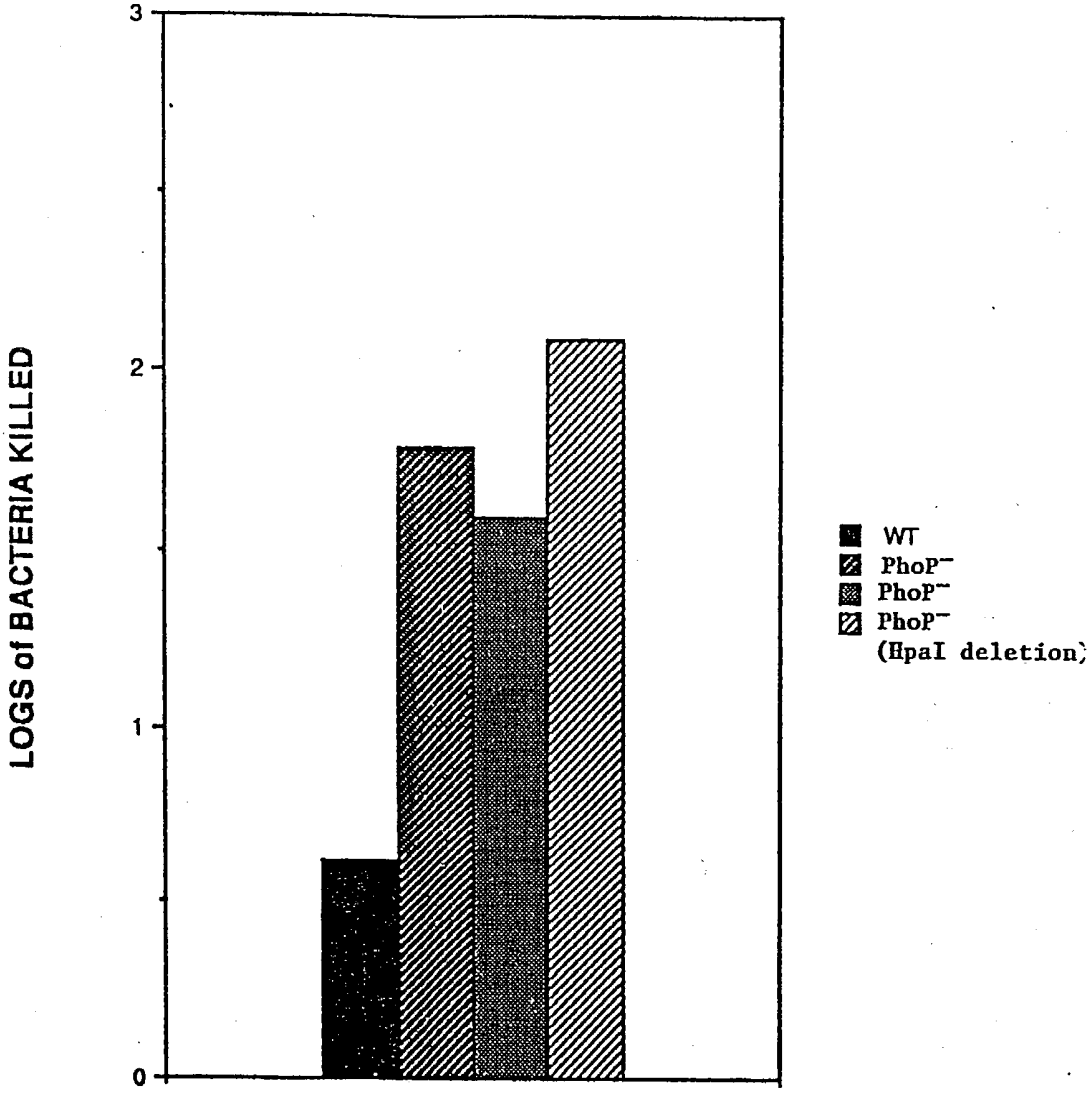

LOGS of BACTERIA KILLED
3
2
1
0
WT
PhoP⁻
PhoP⁻
PhoP⁻
(HpaI deletion)

FIGURE 11

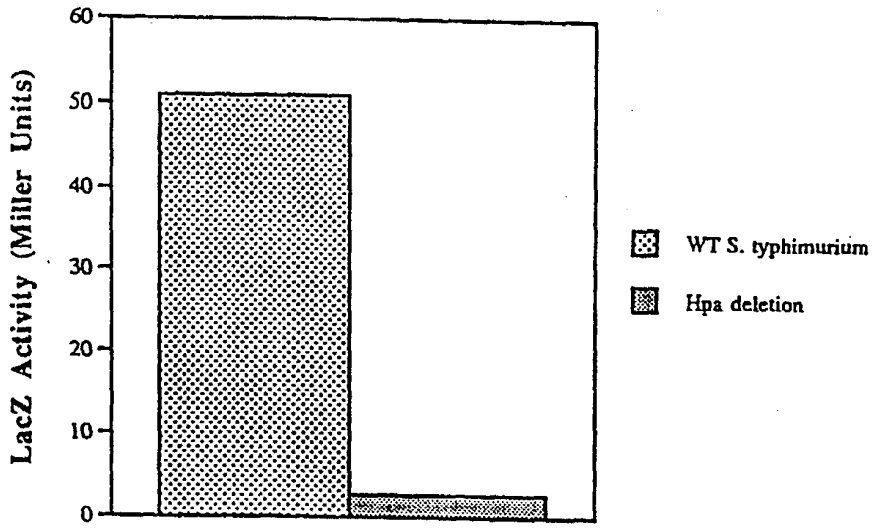

LacZ Activity (Miller Units)
60
50
40
30
20
10
0
WT S. typhimurium
Hpa deletion

FIGURE 12

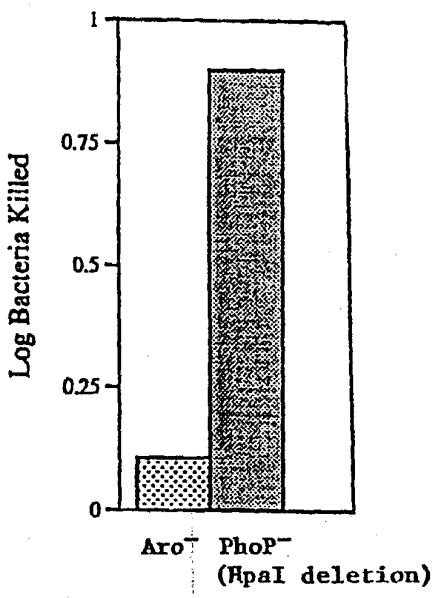


FIGURE 13

FIG. 14 C.

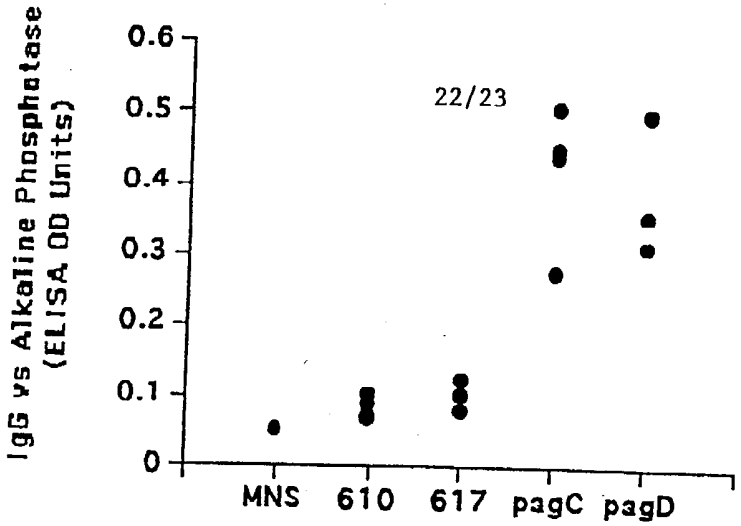
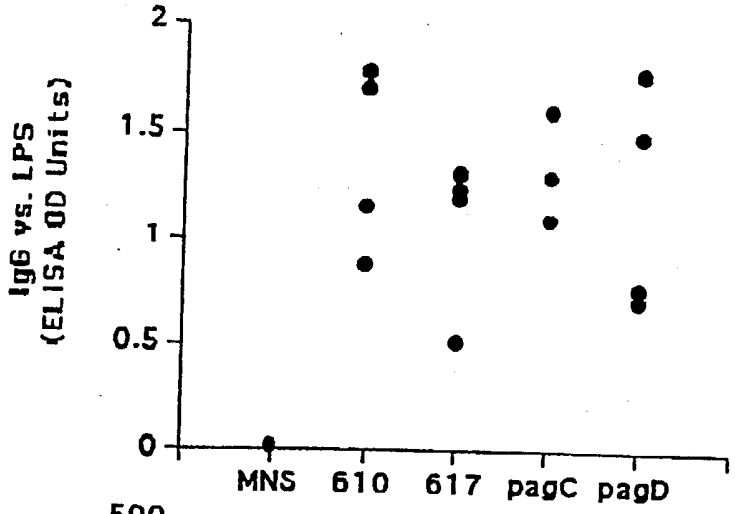
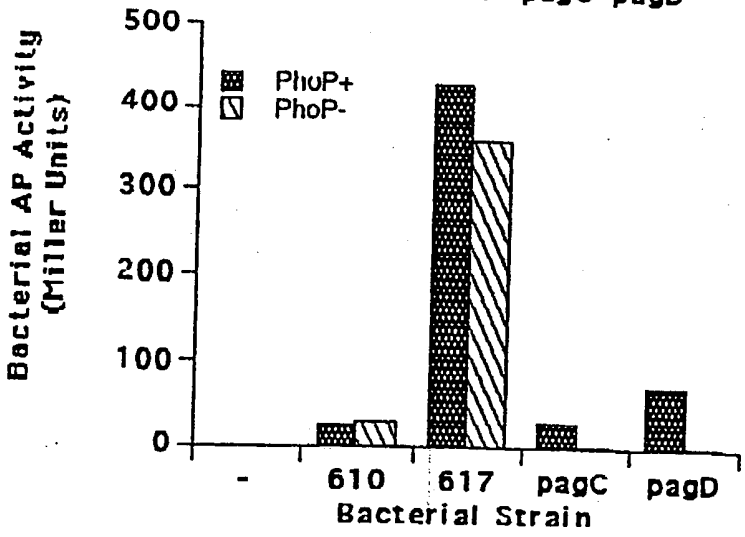


FIG. 14 B.



IG. 14 A.



CATAACAACTCCTTAATACTACTTATTATTTACGGTGTGTTTAAACACCT 50

GCAGTACCGATCCGGCATTCAGTTATCGCCACTATGCCGAATCGACAAAA 100

CCACGAATAATTCACCGCTATCGCTCCTGATGTGTTTACTTCCTGAAAGA 150

TATTTTTACTACCGAAGCACTCTATCGCTCATTTAGGTAACCGGTTCTAC 200

AATGTCATCTAACTTTTATAGATTTGAATGCTAATTTTTCTCACGCATAT 250

ATATTTAACAGAAACCATAAAGTGTTTAGCCACTATAGAACAACAAATCA 300

CCCATGCAACATTTTGATATTTAAAGAGAAAATCTCACAACCACATTAAG 350

AAACTTGACACCGTTCGGCTAAAAACATGTCATTAAGCAAACTCGCCATA 400

TAATCAGAACATATCGCATTGTGCTTCACAGTCCTCACGTGACGCTCCAT 450

CCGCAATACGGTTATATGCCATCGCAGGCGCTGTAATCATATTCACGATG 500

ATGCTTAGCACGCTTTATTCCCGCTCCGATTTAATCTTTTAATATATCTA 550

TCAGTTACAACATTTCTTGTTATATTATAAGAATAGAATCAACACCACAA 600

TTCCAACATAAATATCACCTGTGTTTAGAGAGAATTTACATTCCAAAAAA 650

ATAATAACTAACGCAAATATTGAACACGCGATAAAAAAGTCTATTTCGCT 700

ATAAAACCCATTATTATTAAGAGTGGTTAACTCTTCGTTGAATAAAAAAT 750

GTCAATGACGTTCCATAATTCAGGAGATGAACTTCACAAGTCATTATATA 800

TAACAGGAGGTGCTATG 817 (SEQ ID NO:15)

FIGURE 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07658

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/00, 7/00; C12Q 1/00; A61K 39/02, 39/40
US CL :424/93A, 241.1, 258.1; 435/252.3, 252.8, 320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93A, 241.1, 258.1; 435/252.3, 252.8, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, MEDLINE, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 92/11361 (MILLER ET AL.) 09 JULY 1992, see entire document.	1-196
Y	Vaccine, Vol. 11, No. 2, issued 1993, Miller et al, "The PhoP Virulence Regulon and Live Oral Salmonella Vaccines", pages 122-125, see entire article.	1-196
Y	Molecular Microbiology, Vol. 5, No. 9, issued 1991, Miller, "PhoP/PhoQ: Macrophage-Specific Modulators of *Salmonella* Virulence?", pages 2073-2078, see Abstract on page 2073, and Table 1, page 2074, page 2078.	1-104, 115-123

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 27 SEPTEMBER 1994	Date of mailing of the international search report 07 OCT 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer H. F. SIDBERRY Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07658

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Research Microbiology, Vol. 141, issued 1990, Miller et al, "*Salmonella* Vaccines With Mutations In The *phoP* Virulence Regulon", pages 817-821, see pages 817, 819 and 820.	1-196